Mechanical Stiffness-Defined Matrices for Stem Cell Research and Drug Screening

by

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Abstract

Synthetic polymer matrices or subtrata with tailored elastic properties provide a powerful method to direct biological cell' differentiation and foster cell multiplication. By changing the stiffness of the substrate, human mesenchymal stem cell (MSCs) could be directed along neuronal, muscle, or bone lineages. Matrix elastic modulus can also control anchorage dependent cell's motility, localization, tissue formation and organization. Besides that, synthetic materials such as biodegradable polymers offer a versatile alternative to naturally derived biopolymers. Their mechanical properties can be highly tailored and they are easy to synthesize and shape. Moreover, these platforms can be readily "biologically" fine-tuned toward a particular cell linage by incorporating well-documented parameters, which play crucial roles in cell-extra cellular matrix (ECM) signaling pathway, such as growth factor, surface topology and stimulation signal. Hence, these materials are suitable candidates to develop engineered matrices for stem cell culture, cell manipulating platforms in biological research and drug development.

In this thesis, commercialization aspects of these engineered matrices for stem cell research, cell culture and drug development markets are evaluated both in USA and in Singapore markets. Technological barriers, intellectual property and a preliminary cost model are analyzed. A business plan is presented and discussed for applications in both the stem cell research and the drug screening markets. Although these two markets are ill-defined, both of them are growing rapidly and appear to be very promising. A review of the technology itself led to the conclusion that the matrix is capable of induce anchorage dependent cell into specific lineage but the success rate is not yet quantified and further research need to be done to achieve good reproducibility and to meet the required efficacy of the industry.

Thesis Advisor: Krystyn J. Van Vliet

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I. Introduction

1. Thesis Overview

The main goal of this thesis is to evaluate the potential applications and commercialization feasibility of the elastic modulus engineered matrices for stem cell engineering and drug development.

In the first part of the thesis, the technology is described thoroughly; its current stage of development, its supporting principles and evidence are also presented. In the second part of the thesis, various potential applications are identified. The feasibility of them are subsequently discussed and analyzed. In the third part of the thesis, technological barriers are investigated where requirement of the industry are presented with current competing technologies and the elastic modulus engineered matrices' performance to show both expected opportunities and challenges that the technology is being faced with in the commercialization process. In the later parts of the thesis, intellectual properties, market analysis and business model are investigated and analyzed in details.

2. The Technology

The behavior of cell, including the way they grow, spread and die, depends on numerous factors that the cell receive from the surrounding environments: soluble biochemical signals such as growth factors, genes, hormones as well as mechanical feedbacks such as extracellular strain, stress, external force (illustrated in Fig. 1). Although previously emphasis has been placed on the role of soluble mediators, there is now significant evidence that mechanical factors have the ability to influence cellular status such as inducing differentiation for stem cell, lengthening the culturing lifetime of liver cell, guiding cell motion, localization in vitro.

Several studies indicate that cell actively probe the mechanical properties of their environment and respond with significant changes in cell behaviors including focal adhesion strengthening (1), change in cell morphology (2), cytoskeleton stiffening (3), and elastic modulus guided migration (4). These studies with different mammalian cell have demonstrated that cell do indeed respond to mechanical cues. The hypothesis that stem cell respond to their mechanical microenvironment thus warrants attention.

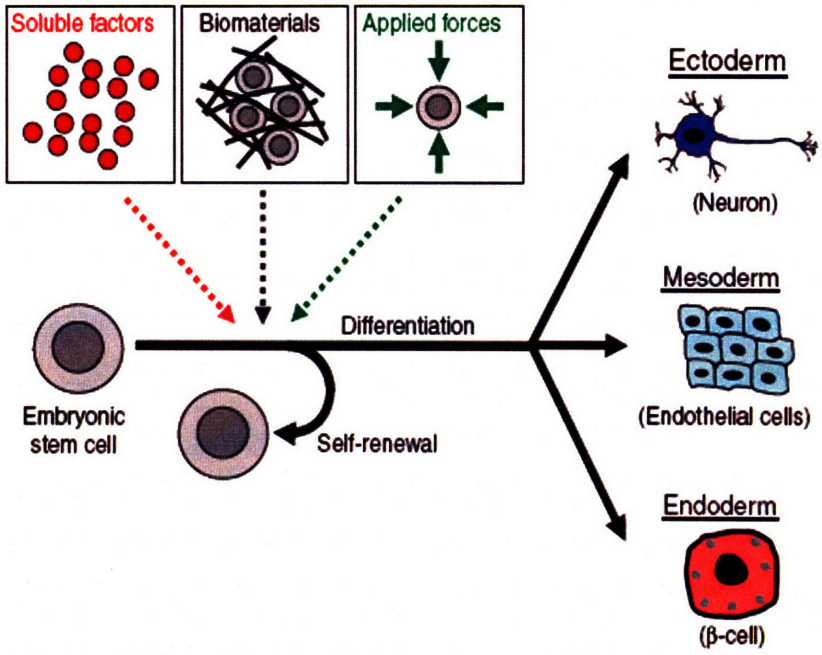


Figure 1: Cues in the microenvironment that affect stem cell fate. This schematic indicates the effect of chemical and physical cues on embryonic stem cell fate, including self-renewal processes and differentiation toward all three germ lineages (5).

Progress in matrix design has also allowed directed differentiation of stem cell into specific lineages (6). The approach of using mechanical property defined matrices to expand and direct differentiation of stem cell provides a promising method to alter stem cell fate in the absence of biological factors, which would be valuable in numerous applications in tissue engineering and drug development. Now, solely by select and regulate the mechanical properties of substrate or tissue microenvironment, we can effectively regulate in vitro differentiation, cell shape and/or lineage commitment of anchorage dependent cell such as mesenchymal stem cell into e.g., neurogenic-, myogenic-, and osteogenic-cell type. Interestingly, inhibitors can be introduced to further regulate differentiation (7).

Besides, cellular adhesion and proliferation are also moderated by mechanical cues. Utilizing microfabrication to control the organization of sheets of cell, regions of high tractional stress corresponded to regions with high concentrations of growth; thus, tissue form is not only a result but also a modulator of tissue growth (8).

a. Technology development

i. Guiding Anchorage Dependent cell' differentiation by matrix elastic modulus

Even though specific ligand-receptor interactions of growth factors and matrix molecules are clearly important for regulating cell, the physical properties of the local microenvironment can also play key roles in determining cellular function and fate (9). Discher et al. conjectured that, the feedback of local matrix stiffness on cell state has been concluded as having important implications for development, differentiation, disease, and regeneration (4). Also, the elastic modulus of the matrix microenvironment has been identified as a new factor regulating stem cell fate (10). By changing the stiffness of the substrate, human mesenchymal stem cell (MSCs) could be directed along neuronal, muscle, or bone lineages as demonstrated in Fig. 3. Compliant matrices favored differentiation of MSCs into neuronal-like cell, moderate stiffness promoted myogenic differentiation, and a rigid matrix stimulated osteogenic differentiation. This behavior is likely due to non-muscle myosin II exerting force through focal adhesions in mechanisms of sensing matrix elastic modulus. Discher et al. suggests that this passive, initial response of MSCs to the microenvironment would be expected from a multipotent stem cell awaiting instruction.

The mechanical forces experienced by a cell are not limited to its contact with surrounding cell, proteins, and surfaces. Nanotechnology and microfabrication technologies enable the directed study of cell-substrate interactions and contribute to these interactions (11). It has been shown that the shape of stem cell regulates the differentiation of human mesenchymal stem cell. Flattened, spread cell committed to osteocytes, whereas round cell became adiptocytes (12).

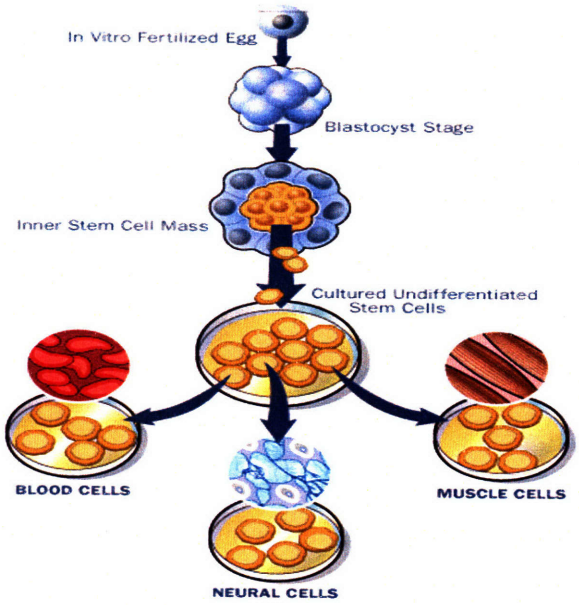


Figure 2: In vitro stem cell differentiation

Concurrent with that, by using scaffolds of collagen, fibronectin (FN) and laminin to alter mechanical properties of scaffolds, embryonic body (EB) formation can be drastically altered. When the elastic modulus was increased from 16 to 34 Pa the formation of EBs was severely inhibited suggesting that the increase in elastic modulus resulted in an inhibition of apoptosis (13). A second possible explanation may be that the denser network of these stiffer gels may have altered ESC growth (14).

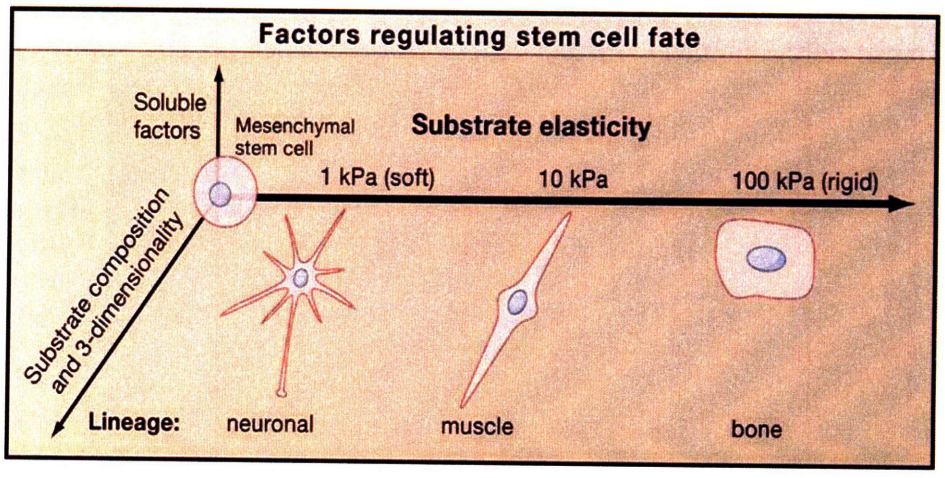


Figure 3: Controlling Stem Cell Fate: multiple factors can influence the differentiation of stem cell, including secreted soluble factors, the elastic modulus or compliance of the matrix substrate, and the biochemical composition and dimensionality of the matrix (9).

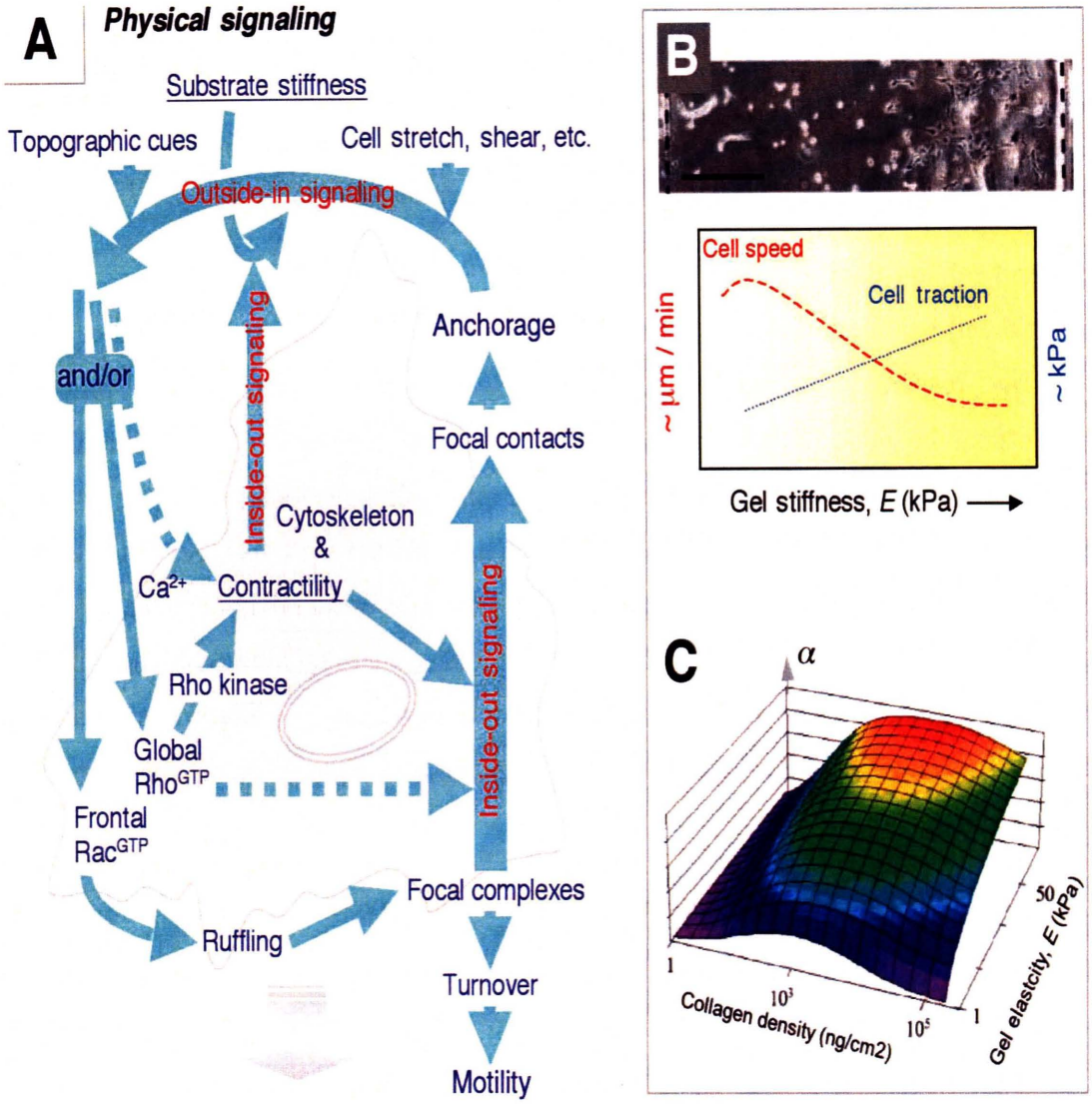


Figure 4: Substrate stiffness influences contractility, motility, and spreading. (A) Interplay of physical and biochemical signals in the feedback of matrix stiffness on contractility and cell signaling. (B) Cell exert less tension on softer, collagen-coated gels but crawl faster, causing an accumulation of cell toward the stiff end of a soft-to-stiff gradient gel. Curves are schematic. (C) Spread area, α, of smooth muscle cell versus ligand density and matrix stiffness, based on measurements fitted by a thermodynamic model. Similar nonlinear responses are also seen for adhesions, cytoskeleton organization, tractions exerted on the substrate, and other cellular processes (4).

In conclusion, while cell have been shown to respond to externally applied forces (15), until the present findings there was no suggestion of a relationship between pluripotent cell differentiation and matrix elastic modulus and how various disease states can complicate the physical remodeling required to decrease elastic modulus to proper, tissue-relevant levels prior to the use of stem-cell based therapies. Thus a need remained in the art to provide a method for regulating

the differentiation of mesenchymal stem cell ("MSCs") into anchorage-dependent cell types. Moreover, similar sensitivity, growth and remodeling principles seem to apply to most anchored cell and by regulating differentiation via contractile mechanisms (7).

ii. Manipulating mature cell in vitro by substrate's elastic modulus

✓ Control cell' motility

Directional cell locomotion is critical in many physiological processes, including morphogenesis, the immune response, and wound healing. It is well known that in these processes cell movements can be guided by gradients of various chemical signals. Yet, little is known about the effects of physical cues such as substrate topography, substrate pore size, elastic modulus... toward cell's motility and migration direction. It has been found that cell showed different morphologies and motility rates when cultured on substrates of identical chemical properties but different rigidities (3). And it has also been long predicted that cell are capable of responding to substrate rigidity through a true active tactile exploration process, by exerting contractile forces and then interpreting the substrate deformation to determine a preferred direction or destination of their movements (3).

In a study conducted by Chun-Min Lo et al. from Boston University (USA), 3T3 fibroblasts were cultured on flexible polyacrylamide sheets coated with type I collagen. A transition in rigidity was introduced in the central region of the sheet by a discontinuity in the concentration of the bis-acrylamide cross-linker. Cell approaching the transition region from the soft side could easily migrate across the boundary, with a concurrent increase in spreading area and traction forces. In contrast, cell migrating from the stiff side turned around or retracted as they reached the boundary. This apparent preference for a stiff substrate is named "durotaxis." In addition to substrate rigidity, Chun-Min Lo et al. discovered that cell movement could also be guided by manipulating the flexible substrate to produce mechanical strains in the front or rear of a polarized cell. It is concluded that changes in tissue rigidity and strain could play an important controlling role in a number of normal and pathological processes involving cell locomotion (3).

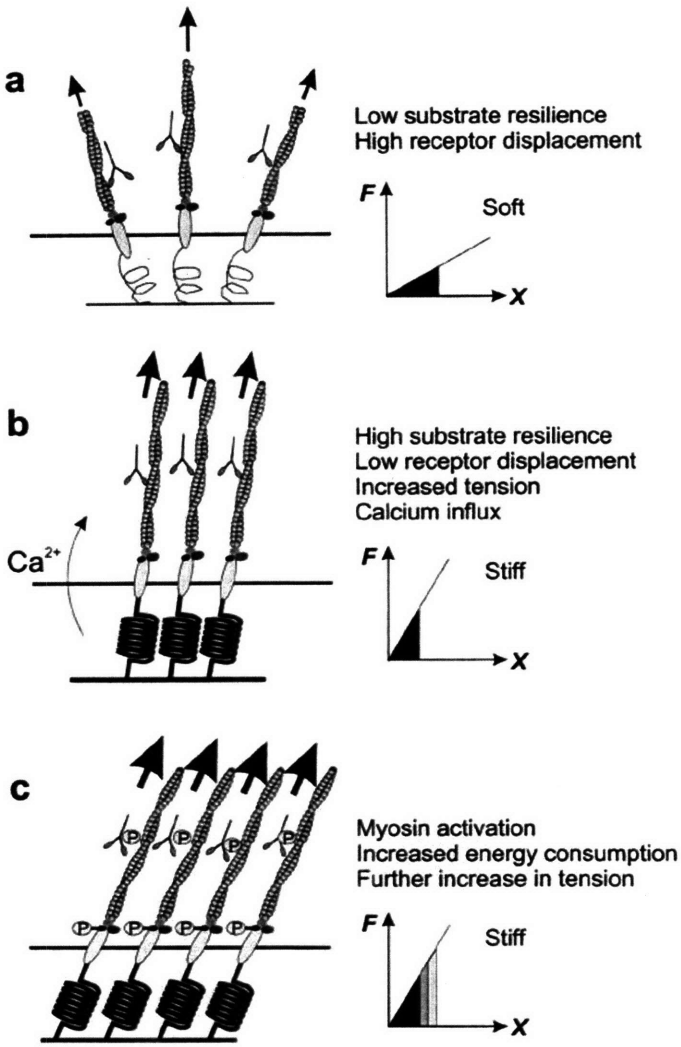


Figure 5: Model for the detection of substrate rigidity. It is assume that initial probing forces are generated by actin-myosin interactions associated with cell-substrate adhesion sites. (a) On soft substrates, the receptorligand complex is mobile and the tension at the anchorage site is weak. With a given energy input (black area under the force-displacement graph), the complex can move over a long distance (x axis). (b) On stiff substrates, equivalent energy consumption (shown as an equivalent black area under the force-displacement graph) causes a higher tension (y axis) and lower displacement of the receptor – ligand complex (x axis). The increase in tension may induce an influx of extracellular calcium through the stress activated channels. (c) The increase in calcium then causes the phosphorylation of myosin, which leads to an increased energy consumption (gray areas under the force-displacement graph) and a further increase in tension. Previous experiments indicated that there is also an increase in tyrosine phosphorylation at the contact site, which may lead to additional forcemodulated responses such as cell growth and gene expression (3).

From these conclusion, the elastic modulus engineered matrices appears to be not only one of the bioresearchers' interests for cell mechanics, cytosckeleton, wound healing study but also potentially be a great tools to manipulate cell movement in vitro.

✓ Specified cell' localization

It has been demonstrated by Darren S. Gray et al. from Department of Biomedical Engineering, Johns Hopkins University School of Medicine in their attempt to repositioning cell by mechanotaxis on surfaces with micropatterned Young's modulus that when cultured on fibronectincoated acrylamide having Young's moduli of 34 kPa and 1.8 kPa, or fibronectin-coated PDMS having moduli of 2.5 MPa and 12 kPa for several days, NIH/3T3 cell and bovine pulmonary arterial endothelial cell accumulated preferentially on stiffer regions of substrates. The migration, not proliferation, of cell in response to mechanical patterning (mechanotaxis) was responsible for the accumulation of cell on stiffer regions. Differential remodeling of extracellular matrix protein on stiff versus compliant regions was observed by subsequent immunofluorescence staining, and may have been responsible for the observed mechanotaxis (16). The results obtained suggest that mechanically patterned substrates might provide a general means to study mechanotaxis.

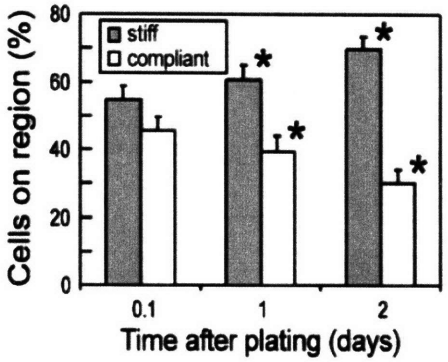


Figure 6: Plot of the percentage of total cell density on stiff and compliant regions as a function of time after plating. Error bars are standard error of the mean (16).

In addition to that, recent advances in microfabrication techniques have enabled substrate fabrication of polymeric cell culture surfaces containing micrometer-scale regions of variable stiffness. Substrates maybe made of either acrylamide or poly-(dimethylsiloxane) and were patterned with 100 μ or 10 μm resolution, respectively. These patterned substrates are ideal candidates for designing cell culturing substrates that are pre-defined in major clusters of cell formation. Also, based on different relative motility of different cell types for the gradient elastic modulus substrate, multiple cell line may be studied at the same time on the same substrates with their positions localized. These also offer a new approach to patterning cell.

✓ Influence tissue formation and organization

The ability of cell to form tissues represents one of the most fundamental issues in biology although the hidden mechanism that triggers cell to adhere to one another in tissues and to migrate once a piece of tissue is planted on culture surfaces still remain elusive. By using substrates of identical chemical composition but different flexibility, Guo et al. have shown that this process is controlled by substrate rigidity: on stiff substrates, cell migrate away from one another and spread on surfaces, whereas on soft substrates they merge to form tissue-like structures (17). It is demonstrated that cell compaction on soft substrates involves a combination of weakened adhesions to the substrate and myosin II-dependent contractile forces that drive cell toward one another. It is also suggested that tissue formation and maintenance is regulated by differential mechanical signals between cell-cell and cell-substrate interactions, which in turn elicit differential contractile forces and adhesions to determine the preferred direction of cell migration and association (17).

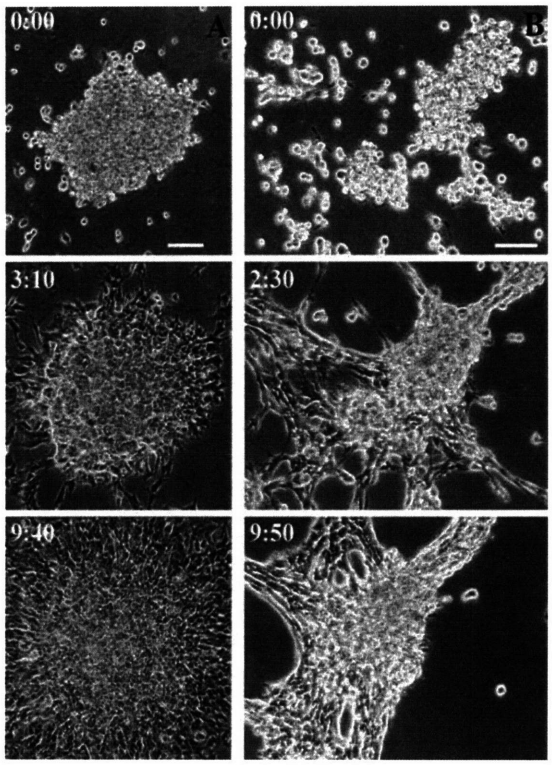


Figure 7: Response of cell-cell associations of cultured fibroblasts to substrate flexibility. 3T3 fibroblast cell aggregates are plated on either stiff (A) or soft (B) substrates. On stiff substrates, cell show the typical scattering behavior as seen on conventional culture dishes (A). In contrast, cell form tissue-like aggregates when plated on soft substrates (B). Time in hours and minutes is indicated. Bar, 100 μm (17).

✓ Lengthening culturing lifetime of Hepatocytes in Vitro by matrix elastic modulus

A major challenge exists in designing materials suitable for supporting liver-derived cell cultures. To attain organ-equivalent levels of tissue function from cultures, the growth substrates must not only be biochemically hepatospecific but also biophysically sensitive to its mechanical nature to achieve a highly differentiated cell phenotype. Physical resistivity of the cellular environment is thought to determine cell shape and, as a result, remodel the internal architecture of intracellular signaling processes. One of the biophysical parameters which are intimately coupled with the outcome of hepatocellular morphogenesis is the substrate mechanical compliance because of the high sensitivity of a hepatocyte to the mechanics of the environment. Hepatocyte morphology is known to be closely linked to cellular functions. As a result, morphogenesis is extremely important to attain organ-equivalent levels of tissue function from *in vitro* cultures. Thus, a puzzle lies in designing materials suitable for supporting liver derived cell that are not only biochemically hepatospecific but also biophysically sensitive to the mechanical nature of hepatocytes to achieve highly differentiated cell phenotype found in a natural liver.

In one study, a unique substrate material system of polyelectrolyte multilayers (PEM) that can be tuned to achieve mechanical compliances of several orders of magnitudes ($E_S = 10^5$ to $E_S = 10^8$ Pa) was employed (18). Further PEM modification effectively changes the surface mechanical compliance, and, thus, hepatocyte morphology and attachment, by controlling pH deposition conditions (pH 2.0, 4.0, and 6.5) and collagen concentrations on different materials (tissue-culture polystyrene, polycarbonate, and Permanox). For all materials, PAH/PAA 4.0/4.0 provided the balance of cellular attachment that appeared neither confluent nor sparse while also promoting a natural hepatocyte phenotype. Interestingly, PEM films were confirmed to be able to effectively mask any inherent substrate material properties. Therefore, the use of PEM modification can be applied to a variety of surfaces and geometries for hepatocyte cultures. PEM and their controlled elastic modulus is therefore demonstrated to be an invaluable tool in optimizing cellular attachment and function and will prove to be essential to future *in vitro* hepatocyte studies.

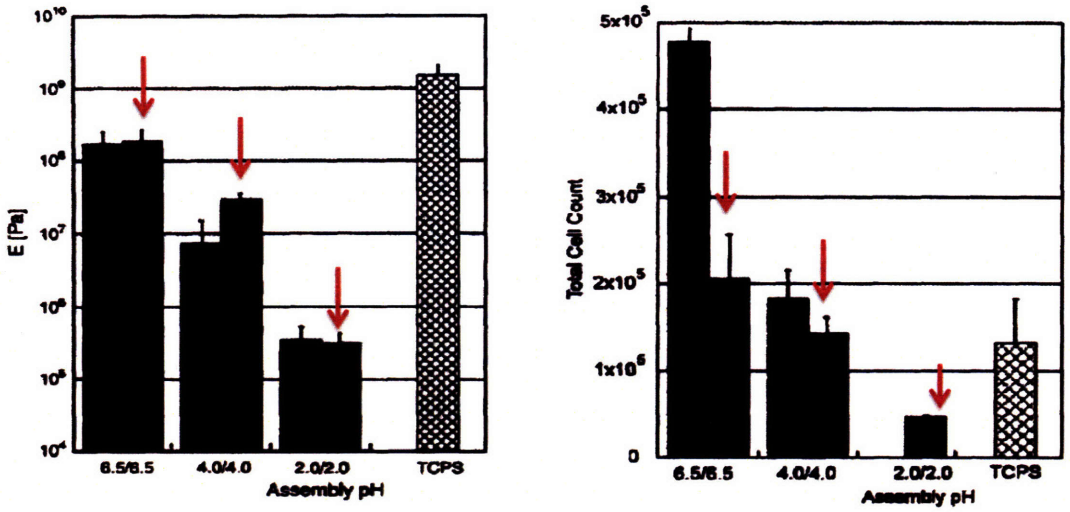


Figure 8: (A) Young's Modulus E, as a function of assembly pH of PAH/PAA solutions, pH = 6.5, 4.0, 2.0. The terminal layer is indicated as PAA or PAH (red arrow on top). (B)Total number of fibroblast cell harvested from 60 mm-diameter Petri dish at seven days post-seeding, as a function of PEM assembly pH (18).

b. Supporting Principles

With the ability to directly control the substrate mechanical compliance of the PEM and other bio polymer matrix complexes, as well as the capability to control degree of cell attachment to the matrix as well as the force interaction between cell and matrix, the technology of using elastic modulus controlled matrix can manipulate and control cell morphology, differentiations and expressions. This section will present some supporting principles available up-to-date to affirm the technology. These discussions, proposed by Discher et al. group, are mainly focus on the differentiation inducing effect of elastic modulus controlled matrices on stem cell. Other effects of defined elastic modulus matrices on handling and manipulating cell are still in preliminary research stage. Their models and supporting principles are still under investigation. Hence, they are not presented in this thesis.

✓ Force and deformation of living cell

There have been quite a number of models developed that explain the reasons that extracellular mechanical signals affect cell states. One model is that cell such as those in bone and endothelium are subjected to specific forces as part of their 'native' physiological environment. Any alteration of such forces is likely to cause a disruption in their normal functioning, thereby producing a diseased state. Another model is that specialized cell, such as the cochlear outer hair cell, realize their functions by converting an electrical or chemical stimulus into a mechanical

force that has a more direct role in cell function (21). Yet another model is that for certain cell, such as muscle cell, a mechanical signal in the form of force or deformation transmitted during an activity, such as exercise, facilitates a function that is not necessarily a mechanical one (22).

Mechanical loading of cell induces deformation and remodelling, which influence many aspects of human health and disease. Also one explanation for the fact that during cell locomotion, the crawling of cells can be altered markedly by the stiffness of the substrate is because cells have the ability to recognize the mechanical environment (for example stiffness) and adjust their behavior (for example direction of motion) accordingly (22).

✓ Matrix stiffness, matrix strain and cell differentiation

In macroscopic view, stiffness demonstrates the ability of a solid tissue to recover its shape in a short time range (in seconds) after mild poking and pinching, or even after sustained compression. At the cellular scale, normal tissue cell probe elastic modulus as they adhere and pull on their surroundings. Biologically, such processes are dependent in part on myosin-based contractility and transcellular adhesions-centered on integrins, cadherins, and perhaps other adhesion molecules-to transmit forces to substrata(7). On the other hand, microenvironments are crucial in stem cell lineage specification and differentiation as cells can 'feel' tissue softness via contractile forces, generated by cross-bridging interactions of actin and myosin filaments. These forces (referred to as traction forces) are transmitted to the substrate, causing wrinkles or strains in thin films or soft gels (19). The cell, in turn, responds to the resistance of the substrate by adjusting its adhesions, cytoskeleton, and overall state, e.g differentiation.

It is suggested that by selecting, designing, or engineering a substrate or tissue microenvironment having an elastic modulus defined by elastic constant E; and introducing the anchorage-dependent cell onto a substrate or into a microenvironment, balance of chemo-mechanical energetics localized to cell adhesions against contractile energetic can be achieved, thereby cell shape and lineage commitment are controlled. Since the cell adhesions area increases linearly with E, larger intracellular deformation occurs on stiffer matrices and larger deformation in the substrate occurs on softer matrices (7). Also, it is proposed that cell differentiation can be further regulated by controlling cell strain, such that there is an inverse relationship between intracellular and extracellular strains so that on stiff matrices, cell strains are large, while matrix strains are

small, and on soft matrices, cell strains are small, while matrix strains are large. The strain thus transfers from outside to in with increasing matrix stiffness, presumably activating different pathways at different strains (10).

Presently, there is no theory or model successfully quantifies effect of matrix mechanical properties such as elastic modulus or strain toward cell differentiation. Discher et al. proposed a model that the free energy depends additionally on the global pre-stress, σ, assumed to act throughout the cell volume V as a global regulator of differentiation. Consequently, when coupled to this, an increase in free concentration of the cells' transducing activator links cooperatively to collagen. Discher et al. concludes that the net result is a lineage commitment probability given by:

$$P_{lineage}(E) = a_0 + a_1 \exp(-\sigma V / k_B T_{eff}) \left[\frac{E^m}{E^m + K^m coll^m} \right]$$

where $k_B T_{eff}$ is effective thermal energy which suggested to be relate more to cytoskeletal stochastics than temperature. K is apparent affinity of lineage-specific component related to collagen (*coll*) with a Hill coefficient m.

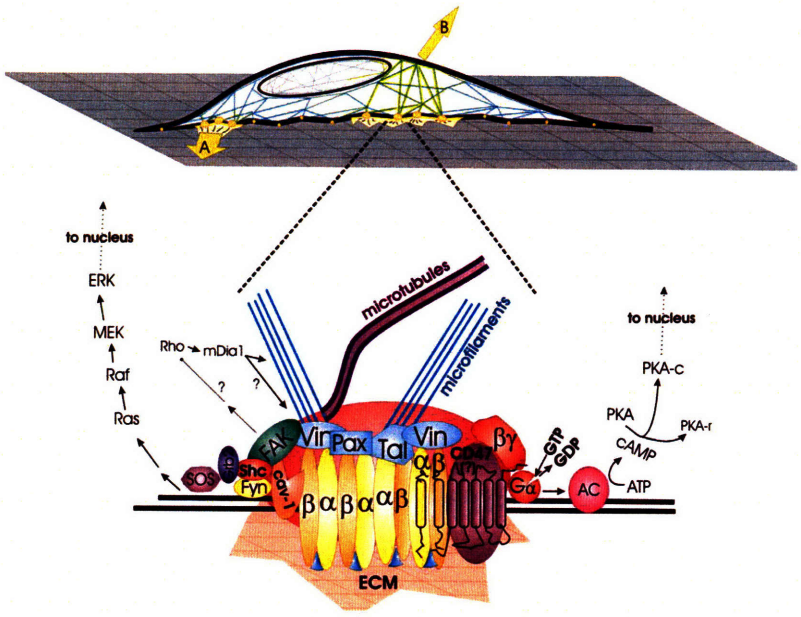


Figure 9: Schematic diagram of how forces applied through the ECM (A) or directly to the cell surface (B) travel to integrin-anchored focal adhesions through matrix attachments or cytoskeletal filaments, respectively. Internally-generated tension and forces transmitted through cell-cell contact similarly reach focal adhesions through the cytoskeleton (20).

II. Potential Applications

There are three main targeted applications for this technology: as means to control stem cell differentiation, as platform for cell handling (assist cell manipulations; maintain cell in appropriate states) and high-throughput whole cell based bioassay for pharmaceutical development. Fig. 10 lists out all the possible application aspects of the elastic modulus defined matrix. It is noted that our matrixes are only targeting at non vitro applications of tissue engineering; it's used as media for cell to growth or to handle and manipulate cell before they are introduced into the body.

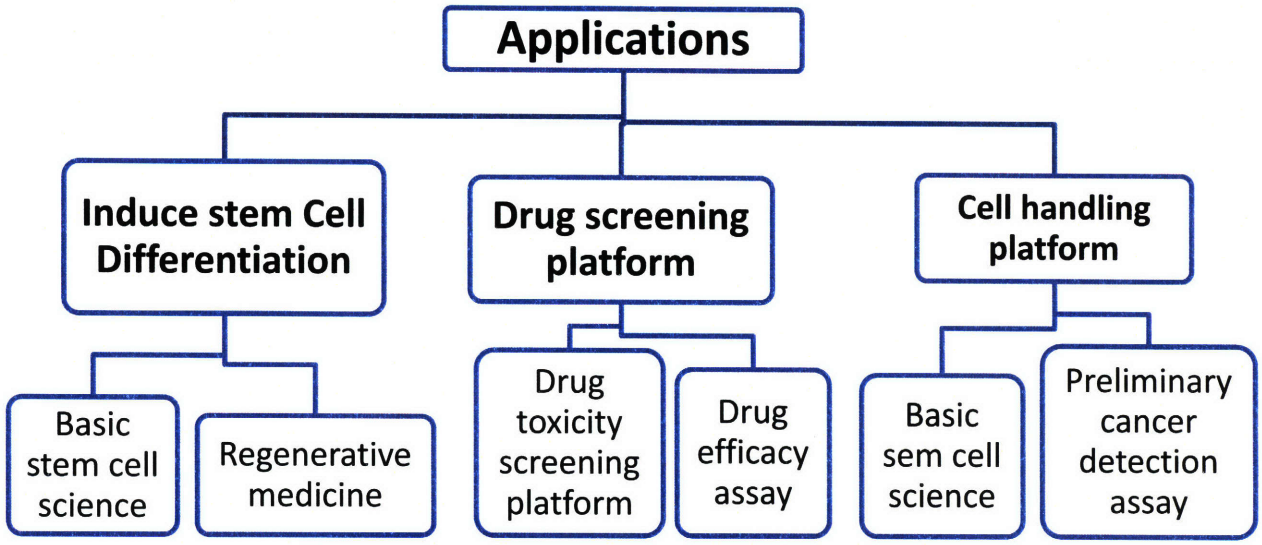


Figure 10: List of potential applications of elastic modulus engineered matrices in bio research and drug development

1. Matrices for stem cell differentiation

Elastic modulus engineered scaffolds can play a number of specific roles in tissue engineering applications using stem cell: as defined systems for stem-cell derivation and expansion, as substrates for clonal expansion of genetically engineered stem cell, or also as factors to facilitate differentiation of stem cell.

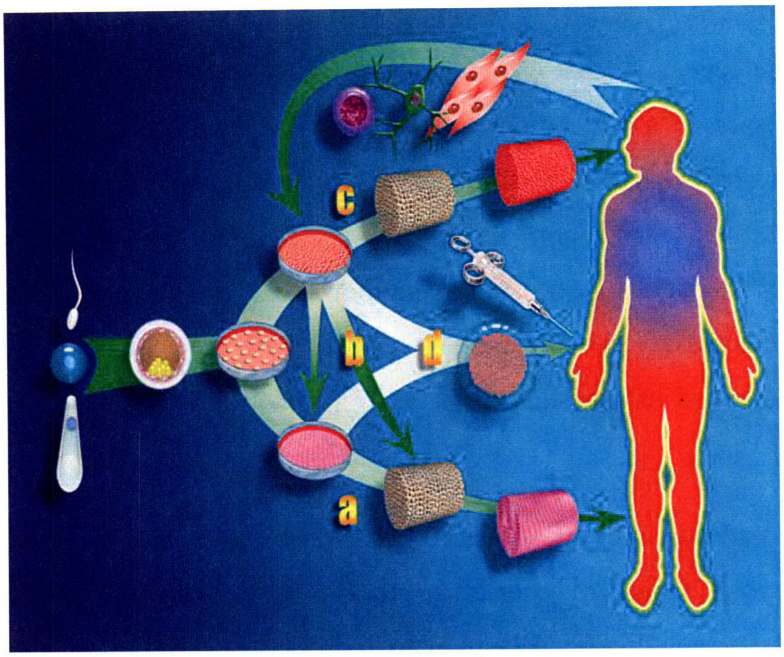


Figure 11: Multiple roles for engineered matrixes/scaffolds in stem cell TE. Biomaterials play different roles at various stages in the application of stem cell to TE. ESCs may be derived from blastocysts obtained by either fertilization or somatic cell nuclear transfer under xeno-free conditions on biomaterial substrates. Derived stem cell can be expanded in culture on biomaterial-based bioreactors. Tissue scaffolds can be tailored according to the specific goals of the intended therapy. (a) Expanded ESCs can be differentiated terminally into mature cell types before seeding into scaffolds to construct tissues or whole organs. Alternatively, expanded stem cell may be partially differentiated into committed tissue progenitors (proto-tissues) that undergo terminal differentiation in seeded scaffolds (b) before or (c) after implantation into the body. In the latter case, the progenitor cell may continue to proliferate and migrate outward from the implanted graft to repair lesioned areas (6).

a. Molecular biology research

The last 15 years have witnessed major advances in the isolation, culture, and the induction of differentiation of stem cell from various sources. Stem cells have now been identified in every major organ and tissue of the human body. Align with these discoveries are intense efforts to understand the molecular mechanisms underlying the decision of stem cell to enter mitotic dormancy, undergo self-renewal, or differentiate terminally. An understanding of these molecular mechanisms would realize tremendous therapeutic potential of stem cell (6).

Stem cell of embryonic origin can be used to analyze in vitro the development from undifferentiated pluripotent cell to terminally differentiated cell types recapitulating processes of early embryonic development. Therefore, these systems represent in vitro alternatives to animal tests for mutagenicity, cytotoxicity and embryotoxicity studies (23).

i. Mutagenicity

Developmental defects may occur when mutagenic or embryotoxic substances interfere with the regulatory processes of proliferation and differentiation at the level of gene and protein expression. Disturbances of these processes may result in abnormal embryogenesis and malformations. Embryonic abnormalities may be caused by mutations acquired at an undifferentiated state or during germ cell maturation. To study mutagenic effects on germ cell in vitro, it would be necessary to comparatively analyze these effects with data on embryonic stem cell or germ cell-derived cell lines (24).

ii. Cytotoxicity

Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity of the cell which may or may not be related directly to cell death, whereas embryotoxicity tests include the analysis of toxic effects of drugs or environmental factors on embryonal cell or on early embryonic developmental stages (23).

iii. Embryotoxicity

In vitro screening systems for embryotoxicity or reproductive toxicity, the effects of test compounds on both, regeneration and differentiation of a given cell population have to be taken into consideration. Pluripotent EC, ES and EG cell fulfill these requirements, and therefore have been included into cytotoxicity and embryotoxicity test procedures and validation studies (23).

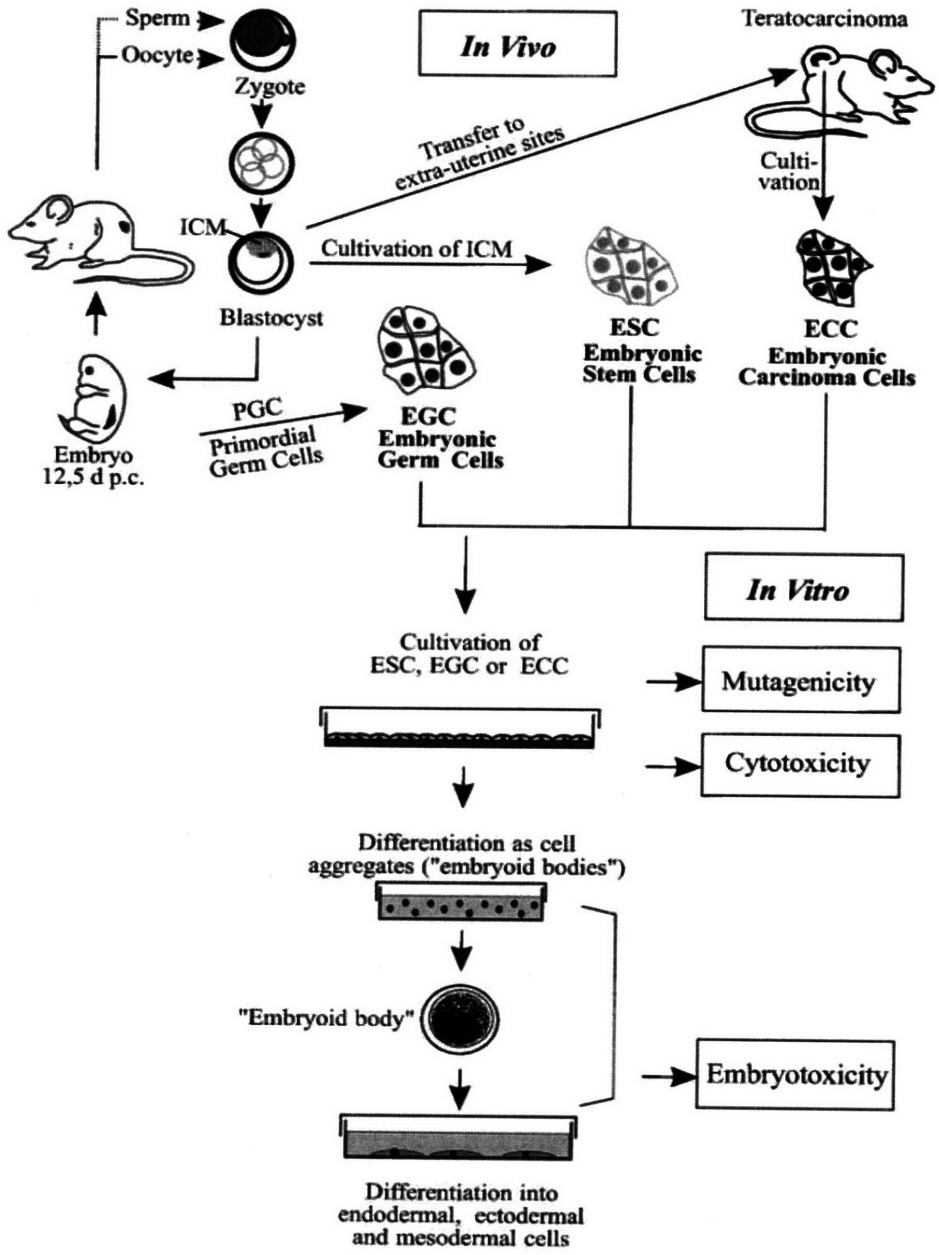


Figure 12: Origin and differentiation potential of embryonic carcinoma (EC), embryonic stem (ES) and embryonic germ (EG) cell, and the use of the ES cell technology for mutagenicity, cytotoxicity and embryotoxicity analyses in vitro (23)

Clearly, innovative methods and developments in engineered matrixes for better control over stem cell culturing will help to establish high-throughput screening analyses on a large scale, to reduce the amount of cellular samples and to save time. Moreover, these methods will result in the identification and characterization of target genes, cell and tissues for teratogenic substances.

b. Regenerative medicine - In vitro synthesis (e.g. epidermis)

Beside in vivo repair of damaged organs and tissue, the promise of cellular therapy lies in generating tissue constructs in vitro for subsequent transplantation. Creating reserves of undifferentiated stem cell and subsequently driving their differentiation to a lineage of choice in an efficient and scalable manner is critical for the ultimate clinical success of cellular therapeutics (14).

Numerous engineered matrixes and scaffolds have proven to enhance osteogenic (25), hematopoietic (26), neural (27), and chondrogenic (28)(29) differentiation. They serve as biointeractive stages promoting cell attachment, proliferation, and organization. Analogous to these matrixes, the elastic modulus engineered substrates with proven ability to induce stem cell differentiation into favorable lineages can serve as regenerative medicine fabrication platform. These elastic modulus defined matrices can also be used in conjunction with other stem cell induced methods (using biochemical soluble factors such as genes, growth factors, hormones or other mechanical signals such as shear stress, intercellular interactions…) to have more drastic net effect toward the stem cell differentiation. Hopefully, with this, more cell products will be supplied to meet the need of current regenerative medicine industry.

2. Cell-based assay for drug screening

Stem cell has significant potential not only in regenerative therapies but also in pharmaceutical development. Yet, in order to translate stem cell research into the clinic, a number of challenges must be addressed. And it's still the development of methods to provide control of stem cell behaviors, including growth and differentiation that remains to be an important challenge. The high throughput development of new bioactive materials and microenvironments will provide important tools to address this problem (11).

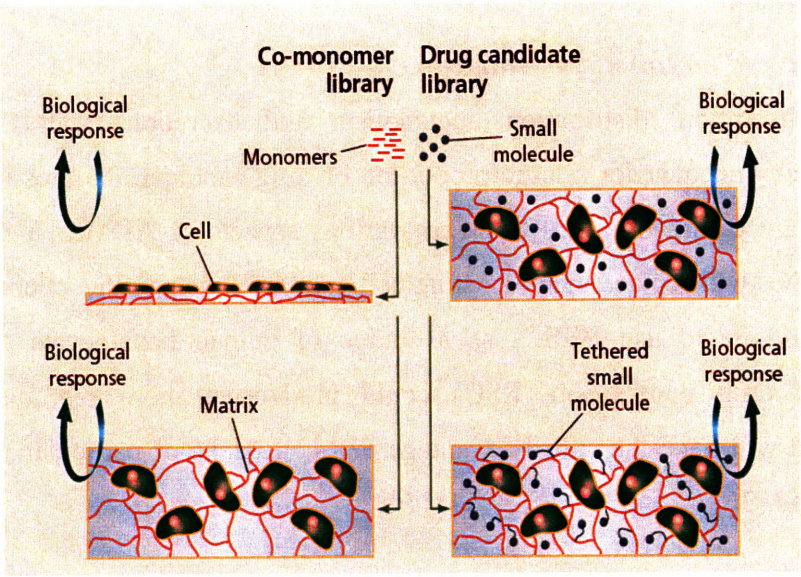


Figure 13: High – throughput screening can affect biomaterials science, and biomaterials science can potentially affect high-throughput screening of drug candidates. (a) Members of a relatively small library of co-monomers are co-polymerized to produce much larger library of polymers. These biomaterials candidates can then be screened for biological activity. (b) The biomaterials is used to provide an extracellular milieu of bimolecular and biophysical signals, which provide a context in which biological responses to members of a large drug candidate library can be monitored. The drug candidates can be soluble within the biomaterial extracellular milieu (upper), or bound to it as is the case with many natural signals in vivo (lower) (34)

a. Target validation

It is clear that cell derived from HESCs should be better models for the development of useful screens than commonly used immortalized cell lines. What remains to be seen is whether assays derived from HESC can be delivered in a cost-effective manner. It is important that pharmaceutical scientists play a role in developing protocols for cell culture models. At present stem cell biologists are focused on understanding the signaling systems and transcription factors that drive differentiation. Pharmaceutical scientists need to help with the characterization, isolation and banking of precursor cell, defining the phenotype and pharmacology of differentiated cell, and on establishing methods to improve the homogeneity of differentiated cell derived from ESCs. It will be particularly important to set appropriate and realistic targets for homogeneity, based on the specific needs and desired outcomes of screening systems.

Although genetic modification is undesirable in cells which are destined for applications in cell therapy, cell developed for uses in screening can be modified to express a reporter gene such as luciferase, to produce very sensitive functional assays.

b. Screening platform

i. Drug transport and metabolism evaluation

Inappropriate absorption, distribution, metabolism and excretion (ADME) together with unexpected toxicity account for a large proportion of drug candidate failures in the clinic. Cell culture models are commonly used to assess various aspects of ADME. It is likely that cell derived from ESCs will replace many existing cell culture models in due course. An immediate application would be to use HESCs as a source of human hepatocytes (35) for in vitro experiments on drug metabolism. ESCs could produce a more reproducible source of hepatocytes, and will have the added advantage that cell banks of particular human genotypes can be established (36).

ii. Pre – clinical drug efficacy testing

Others than biomechanical assay for cancer detection, the elastic modulus defined matrices can also be used to develop pre-clinical drug efficacy testing assay. By measuring the elastic modulus of cell treated with doses of the drug compound of interest, the efficacy of that compound to bring cell back into its normal stiffness state can be evaluate. And this is one of the indications of how efficient or how competent the drug is in treating cancer cell.

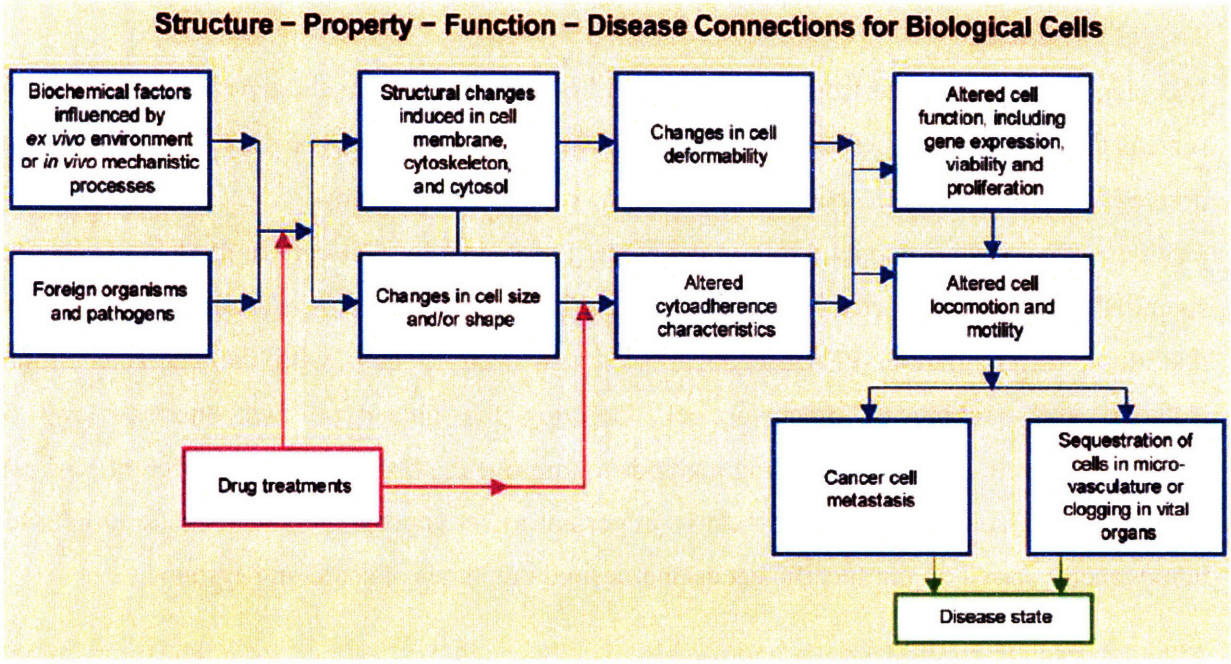


Figure 14: Schematic of chemobiomechanical pathways influencing connections among subcellular structure, cell biomechanics, motility and disease state (32).

iii. Toxicity assessment (toxicogenomics)

The potential to isolate any cell type from stem cell means that in principle it will be possible to study the cellular toxicity of new drug candidates using models for any (or all) tissues and organs. This will lead to an understanding of the influence of the cell phenotype on its susceptibility to toxicity.

Treating specific cell types with chemical or physical agents and measuring their response offers a shortcut to test the toxicity in various organ systems in the adult organism. For example, to evaluate the genotoxicity of a chemical (e.g., drug or pesticide) or a physical agent (e.g., ionizing radiation or non-ionizing electromagnetic radiation) during embryonic development, a large number of animals are being used. As an alternative, use of stem cell lines would be a feasible proposition. Using stem cell lines, efforts are being made to standardize the protocols, which will not only be useful in testing the toxicity of a chemical or a physical agent, but also in the field of drug development, environmental mutagenesis, biomonitoring and other studies (24).

The measurement of gene expression levels upon exposure to a chemical/physical agent can, not only provide information about the mechanism of action of toxicants, but also a sort of "genetic signature" from the pattern of gene expression changes it elicits both in vitro and in vivo. The development of such gene expression signatures would allow the screening of unknown or suspected toxicants on the basis of their similarity to known toxicants. Hence, stem cells are an important new tool for developing unique, in vitro model, with a potential to predict genotoxicity in humans (24).

Human ES cell lines may, prove clinically relevant to the development of safer and more effective drugs for human diseases. For example, ES cell-derived dopaminergic neurons characterized by high survival capacity in vitro could represent a reliable screening system to test physical agents, neurotoxic compounds, or drugs for the treatment of patients suffering from Parkinson disease, a neurodegenerative disorder, caused by a dramatic loss of dopaminergic neurons.

3. Cell handling platform

a. Platform for cell mechanics study

Elastic modulus controlled matrices can be used to construct platforms for cell mechanics researches. The main advantages of these matrices for cell mechanics study platform are lying not only in their simple geometry and surface properties, and their ability to provide mechanical measurements such as stiffness, traction force, adhesion, deformation; but also in their ability to control cell spread, cell localization, movement and orientation in a predicted ways due to their controlled stiffness.

Therefore, elastic modulus defined substrates offer a better platform for cell mechanics study than conventional petri dishes because the defined elastic modulus of the substrate can be utilized to get better control over certain cell states that facilitate mechanical measurement and studies of the cell.

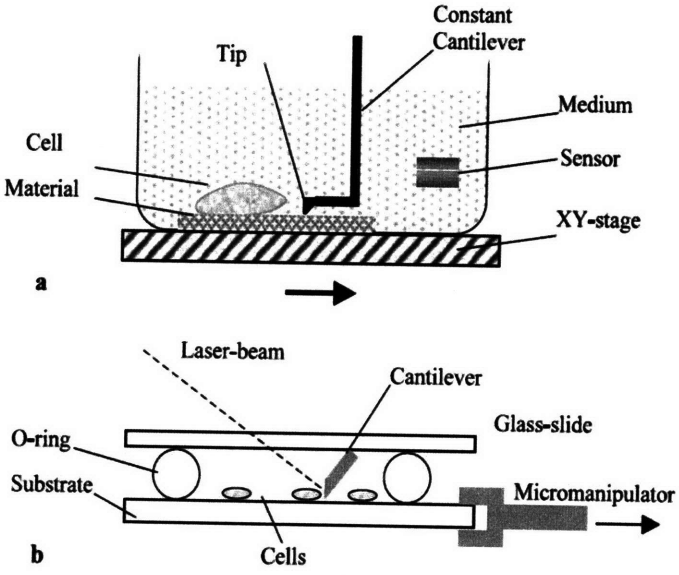


Figure 15: (a) A simplified schematic of the principle of measurement of a cell detachment with the use of shear force; (b) a schematic experimental set-up of a manipulation force microscope, which employs an inclined microcantilever and a laser beam deflection to measure the force (30).

b. Biomechanical assay for cancer cell

Oncologists often diagnose cancer based on a change of tissue stiffness sensed by palpation, yet cancer researchers generally focus on biochemical signaling mechanisms. Tumors are more rigid

because they have a stiffer extracellular matrix (31). Several studies have shown that relative spring constant of pancreatic epithelial cancer cell are decreased significantly with time as the cancer get into metastasis state (32). Some analogous observations have also been reported for other types of cancerous cell such as bladder epithelial cell and breast cell. Hence, this suggests a development of biomechanical assay which can be used in addition to the current conventional biochemical assay for cancer cell detection. There are different ways to probe mechanical stiffness of cell in vitro. Yet, in order to achieve accurate measurements, it is desirable and critical to make sure that the substrates used for cell handlings closely resembles the extracellular matrix environment of the cell in vivo. Also, among all the testing methods, one method uses the sheer force used for peeling the cell out of its adhesion to a reference substrate to calculate the relative stiffness of the cell. The mechanism by which one can measure forces with elastic modulus defined substrate is the correlation of the substrates' deflection versus acting force, which is translated via the substrate's stiffness. The most reliable method of doing that is to attach a cell to a substrate and then to aspirate it with a micropipette. When the cell slips out, the force imposed by the pipette is equal to the force on the substrate.

Hence, the elastic modulus controlled matrix can be potentially served as a biomechanical assay for preliminary cancer detections.

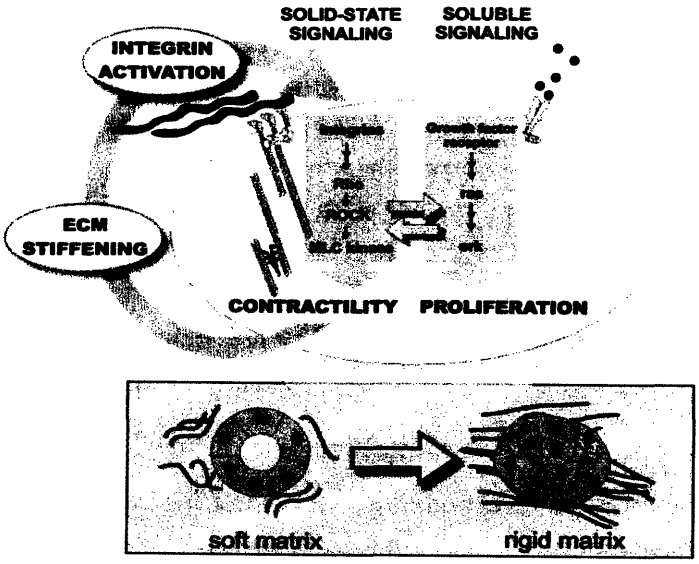


Figure 16: A mechanical autocrine loop that may contribute to cancer development Increases of rigidity in the matrix that better resist cell tensional forces activate integrins, promote focal adhesion assembly, and stimulate the Rho/ROCK

pathway which enhances cell contractility, thereby further increasing matrix stiffness. Because of the crosstalk between the integrin/Rho pathway and the canonical growth factor receptor/ Erk mitogenic signaling cascade, this self-sustaining positive feedback loop may stabilize the undifferentiated proliferative phenotype of mammary epithelial cancer cell and lead to neoplastic disorganization of tissue architecture (31).

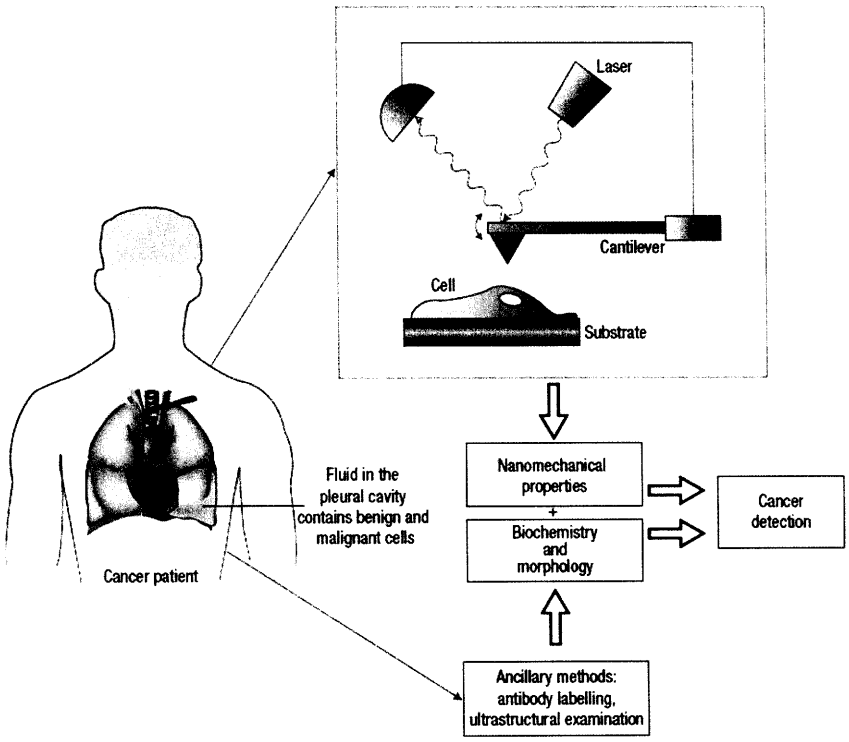
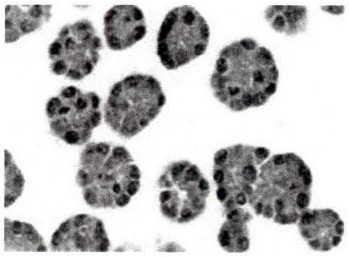
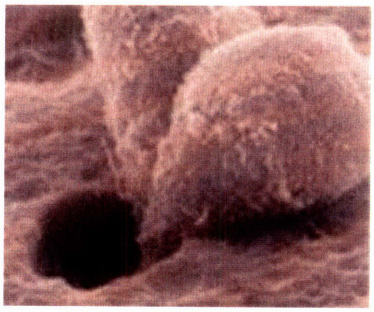
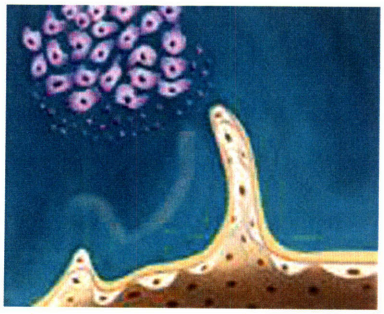


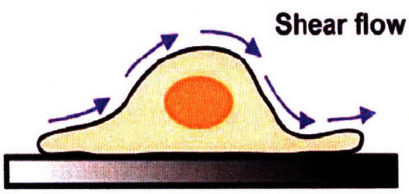
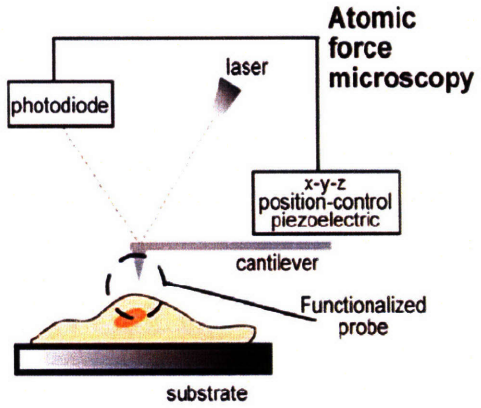
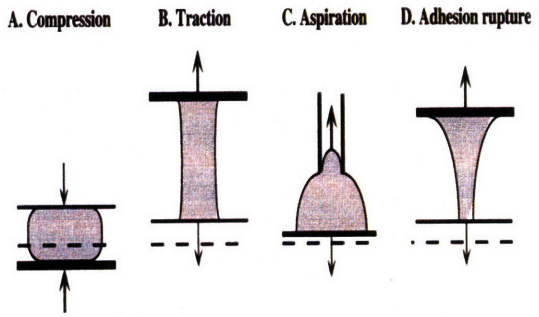
Figure 17: Detecting cancer by probing the elastic properties of cell outside the body. The elastic modulus of benign cell and malignant cancer cell from patients with suspected metastatic cancer were mechanically probed with an atomic force microscope (top path). The elastic stiffness of the cell was used to distinguish cancerous cell from normal ones. Ancillary methods (bottom path), including various labelling and ultrastructural techniques, confirmed the outcomes of the mechanical analysis. Samples were obtained from cavities in the lung, chest and abdomen (33).

4. Conclusion

The main promising applications of this technology are summarized in the table below: Since matrix elastic modulus shows its most dramatic effects in controlling stem cell fates, only the proposed applications that utilize this direction will be evaluated in details in subsequent chapters. For other application directions such as supporting cancer detection platform or cell mechanics study platform, more researches are needed to confirm and quantify the technical success of the matrix elastic modulus's influence before any commercialize evaluation is implemented.

Figure 18: Elastic modulus controlled matrix's proposed applications

Application	Description
Cell Growth and Differentiation Substrate (37) 	The elastic modulus controlled matrix is especially suited for the culture of anchorage dependent cell, such as stem cell and liver cell. It promotes the differentiation of many cell types, including hepatocytes, mammary epithelial, endothelial, smooth muscle cell and neurons
Metabolism/Toxicology Studies (37) 	The elastic modulus controlled matrix can be utilized toconstruct in vitro models of liver cell for drug toxicity studies
Invasion Assays (37) 	The matrix can provides a biologically active basement membrane model for in vitro invasion assay
In Vitro Angiogenesis Assays (37) 	The matrix can serve as a substrate for in vitro liver cell invasion and tube formation assays.

Cell mechanics study platform (31) 	The matrix plays an important role in probing the mechanical properties of cell.
Biomechanical assay for cancer cell (38) 	The matrix can be used as platform to construct biomechanical assay for cancer cell.
Cell manipulation platforms (30) 	The matrix is an efficient tool for mechanical perturbation for individual cell.

It's expected that this technology can achieve some competing characteristics over conventional matrix and other novel matrices. For stem cell growth matrix, with the elastic modulus and cell attachment controlled, we can achieve homogeneity in cell's genome as well as morphology. Hence, cell can grow in phase and we can induce the batch of cell into a specific desired cell line. The technique is cheap since no special reagents or growth factors are needed to go with the matrix. Only basic and conventionally used ECM will do. Furthermore, no animal derived substances presented in the matrix, there is no threat xenogenic effects as in other competitor's products whose matrixes are derived from animals such as *Matrigel*^{TM}.

For high throughput bioassay for drug screening, the competing characteristics that the controlled elastic modulus matrix can obtain are: relevant, quick and accurate response (This will lead to high throughput) and high efficacy (can screen a standard IC50). With this technology, it's expected that we can get a robust whole cell-based bio assay with all relevant cell types, which will potentially gives us faster and more accurate response. Again, the assay is cheap and reproducibility can be obtained if the manufacturing becomes automatic.

The competing advantages of this technology over other conventional methods to direct stem cell differentiation is discussed in details in the next section.

III. Technological Barriers Analysis

In this section, current competing technologies are investigated. Also, industrials requirements are discussed to draw analysis on the technology's entrance barriers in performance's perspectives.

1. Current competing technologies

a. Current stem cell differentiation techniques

Since the isolation of human stem cell (hSC), a large number of groups have shown the ability to differentiate hSC to a variety of lineages with varying levels of competency both in vivo and in vitro. These include both neural cells which appear to be the default pathway for hSC differentiation, and cardiomyocytes, endothelial cell, blood cell, hepatocyte-like cell, insulin-producing clusters.

Basic strategies to induce in vitro differentiation of human embryonic stem cell are:

- Embryonic body (EB) formation: two dimensional monolayer or three-dimensional structure culturing systems. ES cell dissociated from colonies are transferred into suspension cultures, in which ES cell are allowed to aggregate and form spherical three-dimensional structures EBs (summarized in Fig. 19).
- Modification of medium composition: nutrient restriction, reduction of serum concentration, and addition of a growth factor having an impact on gene expression and cell proliferation. Forced proliferation generally results in cell losing their differentiated phenotype, whereas suppressed proliferation results in the initiation of cell differentiation. The control of cell proliferation is related to direct ES cell differentiation towards specific lineages. Growth factors that affect proliferation and survival of specific cell types are often added to a medium to promote differentiation (39) (40) . The following are the typical growth factors often applied:
  - Basic fibroblast growth factor (bFGF),
  - Transforming growth factor β (TGFβ), activin-A
  - Bone morphogenic protein 4 (BMP-4),
  - Hepatocyte growth factor (HGF),
  - Epidermal growth factor (EGF),

  - Nerve growth factor (NGF),
  - And retinoic acid (RA).
- Genetic manipulation of ES cell: forced expression of some transcription factors can direct differentiation of ES cell toward specific lineages. For example, HOXB4 over – expression significantly enhances the hematopoietic potential of mouse ES cell differentiated in vitro (41). GATA-6 and GATA-4 expression in mouse ES cell induces their differentiation into the extra-embryonic endoderm (42). GATA-4 overexpression enhances cardiogenesis and markedly increases the number of terminally differentiated beating cardiomyocytes (43).
- Use of extracellular matrix (ECM) and signaling molecules: interaction between cell and ECM via integrins determines the expression of signaling molecules that affect ES cell differentiation (44)(45). The ECM and integrins collaborate to regulate gene expression associated with cell growth, differentiation, and survival. The developmental fate of differentiating stem cell depends on the complex of growth factors, signaling molecules, and the ECM protein constituting the developmental niche in which the stem cell exist (46). For example, cardiomyocytes are surrounded by a basement membrane consisting of type IV collagen, laminin, fibronectin, and several proteoglycans (47). The ECM applied to a culture system creates a microenvironment in vitro similar to that in vivo.
- Coculture with stromal cell: stromal cell lines support ES cell differentiation. The stromal cell line OP9, which is derived from newborn mouse calvaria, supports hematogenesis, and the bone-marrow-derived stromal cell line ST2 producing macrophage colony-stimulating factor (MCSF) supports osteoclastogenesis from ES cell. The preadipose cell line PA6 promotes neural differentiation of mouse ES cell, and also supports dopaminergic differentiation of human ES cell.

All these approaches have specific advantages and disadvantages, and have been used to generate a broad spectrum of cell types differentiated from ES cell (48). However, there is little or no data on the efficiencies of those methods or a comparison of them, albeit anecdotal accounts suggest that the efficiencies are very low (in the order of single digit percentages). Taking the example of neural differentiation for potential application in Parkinson's Disease, one report has shown that PA6 stromal cell (Zeng et al. 2004) can generate dopaminergic neural cell, while another group has achieved this same lineage via the formation of neurospheres in serum-

free culture (Schulz et al. 2004). In both cases, the time of differentiation was in the order of several weeks (49).

Among the above approaches, inducing EB formation is the most common method used for in vitro differentiation of both mouse and human ES cell. EBs are a powerful tool for studying the differentiation of ES cell into specific and desired cell types.

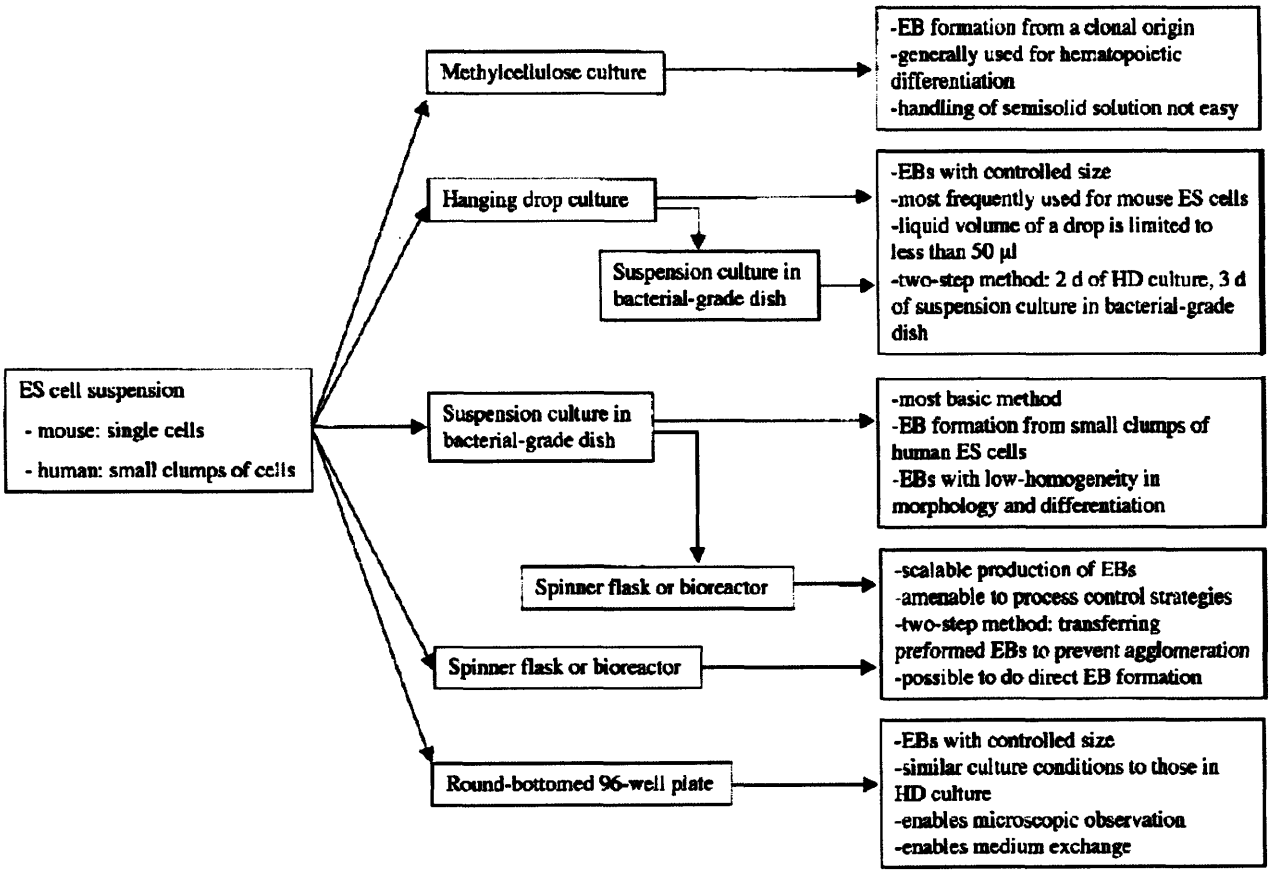


Figure 19: Outlines of methods for inducing EB formation (50).

The technology of using matrix elastic modulus to direct stem cell differentiation is not in direct competition with all of those technologies that are described above. In fact, this technology can be used in conjunction with some of those technologies, for example, to create a differentiation system with controlled matrix stiffness, defined culture reagents and some relevant co-culturing stromal cell to achieve combined effects of all these influencing factors to stem cell differentiation process. In a closer look, using matrix of defined elastic modulus is only in competition with those techniques that use other methods or other materials as substrates for

stem cell culturing such as using bacterial grade dish, petri dish, hydrogel, etc. Most of these current competing materials/ techniques are discussed thoroughly in the next section of this report.

b. Current competing biomaterials as instructive extracellular microenvironments for controlled cell manipulations

Current competing biomaterials that serve as substrate for stem cell differentiation and cell culturing/manipulation can be divided into three main groups: natural materials, synthetic materials and nano-patterned materials.

i. Natural Materials

Table 3 compares currently used natural materials in stem cell culture. A number of other natural materials have been used to support the differentiation for hESCs that include agarose, alginate, hyaluronic acid, gelatin, fibrin glue, and acellular tissue matrices.

- Collagen is the main component of native ECM and cell interact with collagen through integrin binding-mediated interactions. Collagen has long been utilized as a natural biomaterial due to its low immunogenicity. It has been shown that high concentrations of collagen gel inhibited EB apoptosis and enhanced differentiation (46). Addition of fibronectin to the collagen gel preferentially stimulated ES cell differentiation into endothelial cell, leading to vascularization, while addition of laminin favored ES cell differentiation into beating cardiomyocytes (46).
- Gelatin is a porous denatured collagen scaffold, and it has been used for tissue engineering applications due to its biocompatibility.
- Hyaluronan is a high molecular weight polymer having disaccharide unit glucuronic acid and acetyl-glucosamine. HA binds specifically to proteins in the ECM, on the cell surface, and within the cellular cytosol, and thus has roles in a number of different physiological roles such cartilage matrix stabilization, angiogenesis, cell mobility, inflammation regulation, and growth factor action. Currently, HA-based biomaterials have been utilized to support differentiation of stem cell in combination with growth factors or other ECM components.
- Matrigel™, a product currently available commercially, is comprised of a variety of ECM components including laminin, collagen IV, and heparan sulfate proteoglycans and has been used extensively in cell culture. It has also been used to support endothelial differentiation of

ESCs and for promoting the development of glandular- and tubular-like structures from differentiating ES cell (51). Matrigel is less adhesive than collagen and has been shown to support efficient aggregation of ES cell and further differentiation into mesoderm and endoderm lineages (52). However, Matrigel contains a series of unknown proteins, and therefore may not be an appropriate microenvironment for lineage-specific differentiation of ES cell. Hence, in technical view, elastic modulus defined substrates for stem cell differentiation appears to be more competitive over Matrigel™.

- Another biomaterial that has been explored for stem cell differentiation is alginate. Alginate is derived from seaweed, and in presence of a divalent cation such as Ca^{2+}, forms an ionically crosslinked hydrogel. Alginate-based hydrogels in combination with oligochitosan have been shown to support ES cell growth. Additionally, alginate hydrogels demonstrated to be conducive for ES cell differentiation into hepatic lineage (52).

Generally speaking, natural biomaterials may provide efficient adhesion sites for attachment and a wide range of biological signals. Even though these natural scaffolds have been utilized for differentiation and attachment of hESCs, use of naturally-derived biomaterials has been limited to in vitro differentiation application of ES cell due to their weak mechanical properties and regulatory manufacturing difficulties. Major disadvantages of using natural materials over synthetic materials are limited control over physico-chemical properties, difficult to modify degradation rates, difficulty in sterilization and purification as well as pathogen/viral issues when isolating from different sources. Fig. 20 makes a quick comparison of natural and synthetic materials in using as cell culturing scaffolds.

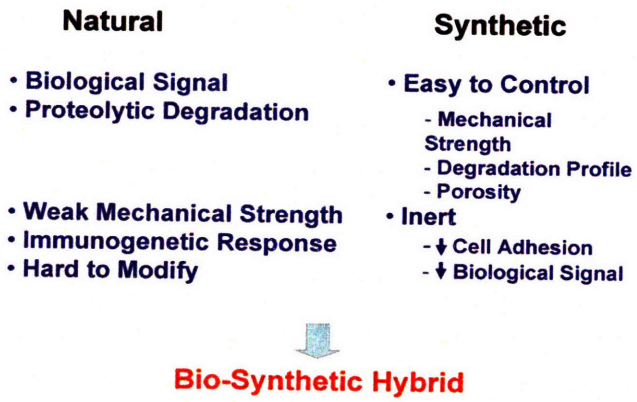


Figure 20: Bio-synthetic scaffolds combine suitable mechanical properties and biological signals that mimic the natural ECM for stem cell-based tissue engineering (52).

Table 1: Application of natural biomaterials in stem cell culture (14)

Biomaterial	Chemical modifications	Application in stem cell culture	Cell source
Matrigel™		Cell culture applications	
		Vascularization	mADSC
Fibrin	Addition of growth factors	Neural differentiation	mESC
		Cell culture	mESC
		Chondrogenesis	hMSC
Hyaff® (hyaluron)		Ligament formation	Sheep MSC
Hyaluron		Maintenance of pluripotency	hESC
	Addition of soluble growth factors	Lineage specific differentiation	
	Modified with photoreactive groups	Cell proliferation	hESC
	Application of hyaluronidase	Cell removal	
Silk fibroin		Medical suture material	N/A
Chitosan with coralline		Osteogenesis	Murine MSC
Hydroxyapatite (HA)		Osteogenesis	mESC
Alginate		Cell encapsulation and differentiation	ESC
Pullulan, Dextran, and Fucoidan	Addition of poly-L-lysine cross-linked with sodium trimetaphosphate	Hepatic possible applications to vascular repair	mESC hESC
Collagen Type II		Chondrogenesis	hMSC
Collagen microbeads		Cell expansion and viability, Hematopoiesis	hUCBC
Collagen	Addition of HA	MSC seeding and proliferation	Rat MSC
	Addition of crosslinked chitin and PLA	Cell attachment	hMSC
	Addition of recombinant human BMP-2 (rhBMP-2)	Osteogenesis	Porcine MSC
Silk		Osteogenesis	hMSC
		Chondrogenesis	hMSC
Chitosan	Conjugation of hydroxybutyl groups	Potential degenerative disk therapies	hMSC

ii. Synthetic Materials

Table 2 summarizes popular synthetic materials used in stem cell culturing. PEG-based hydrogels are ideal as tissue engineering scaffolds due to their high water content, elastic modulus, biocompatibility, and their ability to permit diffusion of nutrients and bioactive molecules. PEG-based hydrogels, however, are bio-inert and do not interact with the cell and therefore various cell-interacting components need to be incorporated to improve their bioactivity. These components include extracellular matrix molecules, small peptides, and glycoproteins.

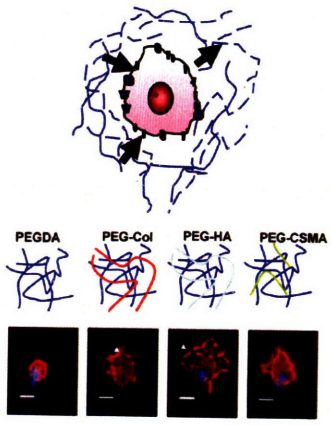


Figure 21: The extracellular microenvironment plays a significant role in controlling cellular behavior. PEG-based hydrogels can mimic natural ECMs both biochemically and biophysically by polymerizing the hydrogels with exogenous ECM components. Distinct cellular morphologies were induced by the various extracellular microenvironments. Actin: Phalloidin (Red), Nucleus: DAPI (Blue). Bar=10 μm (52). Yet, there remains a major drawback is that PEG is bio – inert.

Beside hydrogels, a number of polymers have been microfabricated to develop bioactive, biodegradable, porous, mechanically supportive scaffolds for stem cell differentiation and tissue formation both in vitro and in vivo. One of the advantages of utilizing completely synthetic biomaterial is that their properties-mechanical strength, porosity, degradation profile, and biologically active sites-can be molecularly tailored. Most commonly used synthetic biomaterials are poly (ethylene glycol) (PEG), poly (vinyl alcohol) (PVA), poly (lactic acid) (PLA), poly (lactic-co-glycolic acid) (PLGA), poly (hydroxyl ethyl methacrylate) (PHEMA), and poly (anhydride) (53). Novel synthetic biomaterials have been demonstrated to allow hESC adhesion and guided differentiation toward a desired lineage (53). Yet, the main issue is their poor inherent bioactivity (e.g. PEG), acidic by-products (e.g. PLA or PLGA), etc.

Table 2: Application of synthetic biomaterials in stem cell culture (14)

Biomaterial	Chemical modifications	Application in stem cell culture	Cell source
poly(ε-caprolactone) (PCL)		Cell propagation	mESC
	Addition of adipogenic promoting factors	Adipogenesis	mESC
		Adipogenesis	hMSC
		Chondrogenesis and Osteogenesis	Rat MSC
Poly(L-lactic acid) (PLLA)		Hematopoiesis	mESC
Polyglycolic acid (PGA), polylactic acid, (PLA), polylactide-*co*-glycolide (PLGA)		Cell proliferation and 3-D organization	hESC
Poly(ethylene glycol) diacrylates (PEGDA) hydrogels		Cell and bio-chemical molecule encapsulation	Goat MSC hMSC
	Addition of glucosamine	Chondrogenesis	mESC
	Incorporation of RGD-PEG-acrylates	Cell viability, Osteogenesis	hMSC
	Modified with RGD	Chondrogenesis	hESC
	Release of dexamethasone	Osteogenesis	hMSC
poly(N-isopropylacrylamide-*co*-acrylic acid) [p(NIPAAm-*co*-AAc)]	Incorporated into a photocrosslinked hydrogel with a metalloproteinase sensitive peptide	Cell self-renewal and maintenance	hESC
PEGDA scaffolds	Incorporation of methacrylic acid	Osteogenesis	Murine MSC
poly(6-aminohexyl phosphate acryloyl) (PPE-HA-acryl)		Cell and bio-chemical molecule encapsulation	Goat MSC
Polyethylene terephthalate (PET)		Cell seeding, proliferation, and aggregation	Rat MSC hMSC hHSC
	Conjugated with FN	CD34+ proliferation	hHSC
Poly(ethylene glycol) (PEG) hydrogel	Exposed to TGF-β	Chondrogenesis	mESC
	Addition of a phosphoester	Osteogenesis	Goat MSC

Biomaterial	Chemical modifications	Application in stem cell culture	Cell source
Biphasic calcium phosphate(Triosite™)		Osteogenesis	hMSC
Titanium		Cell attachment and proliferation	Rat MSC
	Differentiation media	Osteogenesis	Rat MSC
	Coated with RGD	MSC attachment	Rat MSC
Surface modification with TiO_2		Cell adherence	Murine MSC
Surface modification with TiO_2		Cell adherence	hMSC
Tantalum (Cytomatrix)		Hematopoiesis	mESC
PPE-HA-acrl	Increase acrylated-PEG	Osteogenesis	Goat MSC
sIPN	p(NIPAAm-*col*-AAc) crosslinked with Gln-Pro-Gln-GLY-Leu-Ala-Lys-NH_2 and functionalized with p(AAc) and RGD complexes	Cell propagation	hESC
Oligo(PEG-fumarate) hydrogels	Modified with osteopontin-derived peptide	Osteoblast migration	Rat MSC
	Modified with RGD	Cell attachment	Rat MSC
Gelatin	Coupled with azidophenyl groups	Cell growth	mESC
Magnetic microbeads		T cell formation	mHSC
Poly(α-hydroxyl ester) scaffolds	Treated with potassium hydroxide	Cell growth	mESC
Poly(L-lactide-*co*-ε-caprolactone) films	Coated with FN	ESC adherence	hADSC
PEG-PLGA polymer blends	Encapsulation of recombinant human TGF-β1	MSC proliferation and osteogenesis	Rat MSC
PDMS molds	Seeded with MEFs	Cell viability and proliferation	hESC
Microwell array system		Create spatially uniform aggregates of undifferentiated cells	hESC
HA microwell system		Cell viability, controlled cell patterning and shaping	mESC

iii. Nano patterned Biomaterials

Natural ECM is constructed through self-assembly of many nanofibrillar proteins secreted by cell, e.g., collagen fibrils. The normal cell environment is comprised of a complex network of extracellular matrix molecules with nano–micro scale dimensions. Though the aforementioned biomimetic hydrogels and porous scaffolds have been fairly successful in providing 3D structural support to cell, they fail to mimic the spatial dimensions of the ECM. In addition, cellular phenotype and differentiation can be profoundly influenced by the diameter of fibrous scaffolds. To this end, the latest efforts in scaffold research have focused on developing biomaterials with nanostructures (54).

Several groups have demonstrated that nanotopography has the ability to enhance differentiation of progenitor cell into their programmed pathway by studying on differentiation and proliferation of hMSCs on nanogratings. Nevertheless, they also pointed out that novelty of this work lies in the application of nanotopography to direct adult stem cell to differentiate into a non-default pathway (Fig. 22). Because of the availability MSCs, their differentiation to functional neurons would be of interest to many for cell therapy. Also of significance is the finding that the topographical cue has to be at the nanoscale in order to exert a significant influence on the directed differentiation. The results demonstrated in a systematic manner that nanopattern, in comparison to micropattern, exerted a significantly stronger effect on stem cell behavior in terms of morphology, proliferation and differentiation (55).

General speaking, the technology of using nano-patterned substrate to control stem cell' differentiation is still in development stage and it is unlikely that it can transform into novel products into the stem cell research market or the drug screening market. Moreover, compared to this technology, the technology using elastic modulus defined substrates to direct stem cell' differentiation appears to be more specific. Since using nano grating substrate to induce cell differentiation complicate identification of possible contributions of substrate stiffness. In contrast, tissue level matrix stiffness is distinct and shown in sparse cultures to exert very strong effects on the lineage specification and commitment of naive MSCs, as evident in cell morphology, transcript profiles, marker proteins, and the stability of responses (10). Therefore, both technologically and in business sense, using matrix elastic modulus to direct stem cell turns out to be more competitive technology.

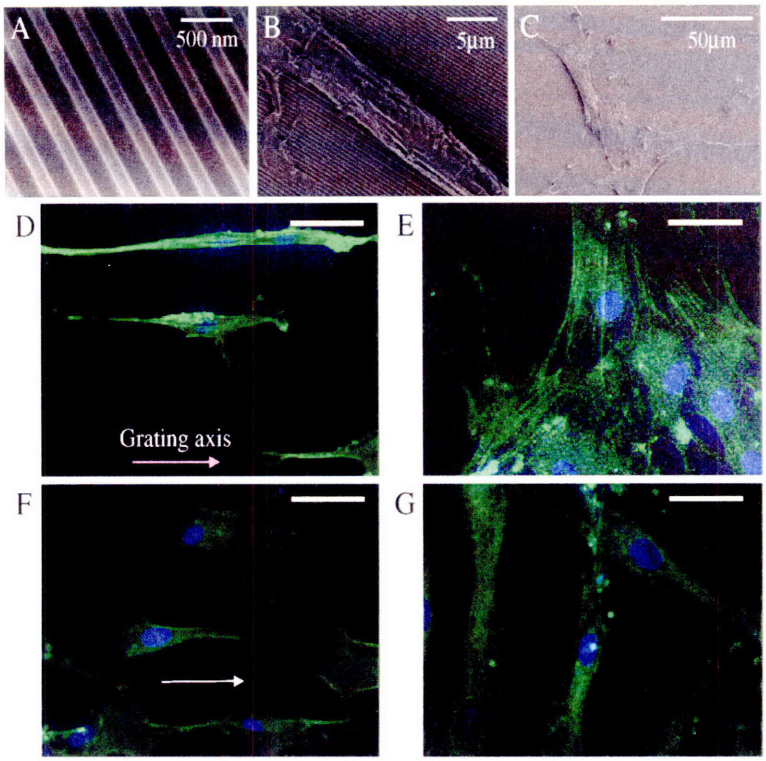


Figure 22: Changes in morphology and proliferation of human mesenchymal stem cell (hMSCs) cultured on nanogratings. Scanning electron micrographs of (A) PDMS nanopatterned by replica molding; hMSCs cultured on (B) nanopatterned PDMS and (C) unpatterned PDMS. Confocal micrographs of F-actin-stained hMSCs on (D) nanopatterned PDMS and (E) unpatterned PDPS in hMSC proliferation medium; (F) nanopatterned PDMS and (G) unpatterned PDMS cultured in presence of 1 μM of retinoic acid (RA). Scale bar=500 nm for A, 5 μm for B, 50 μm for C–G (55).

2. Industrial Requirements and the technology's proposal materials choices

a. Industrial requirements

Industrial requirements for stem cell differentiation systems' efficiency varies with the usage of the final cell products.

Differentiation of stem cell prior to transplantation is very critical, because undifferentiated ES cell may cause teratoma formation in vivo. Therefore, the potential use of ES cell to replace functional loss of particular tissues depends largely on efficient differentiation protocols to derive tissue-specific progenitor cell without any detrimental in vivo side effects. The technology of using matrix elastic modulus is still in innovative stage; it is not mature enough both in technological efficiency and technology capacity. For technological efficacy, reports show that

the technology is capable of driving more than 75% of the cell population into neural lineage, specificity for bone lineage over other lineages in bone-like matrix stiffness may achieve up to 8 times (10) with the same culturing conditions. Yet, it is still far behind the critical requirements of products for vivo usage, where 100% of the cells need to be of the desired lineage. Therefore, we expect that the technology is more suitable to be applied in producing cell for vitro usages which have less stringent requirements over performance. That notion reaffirms the choices of two targeting market which are for stem cell research and drug development.

- For stem cell research market, the market is highly segmented. There are a variety of cell type needed and but the quantity of demand is not large. Currently, the number of products for cell culturing substrates is quite large and the technologies applied are quite versatile (using nanofibers, nanograting materials, 3D hydrogels, etc) but their performance is not well-documented. They also lack systematic evidence and theories to support their clear influence over stem cell growth and differentiation if any.
- For drug development, it is highly desirable to get a fast and accurate response for the whole cell based assay. Hence, the quality of demand is large, so as the quantity. But the cell types needed are limited; few of the cell types normally used are liver cell, neural or kidney cell. There has been no technology emerging as big player as substrate for whole cell based bioassay in the drug screening market.

Due to the vast possibilities of human stem cell, it is envisaged that the types of manufacturing processes to produce a particular lineage will be as varied and complex as the property of the target tissue. Exploiting the expansion capabilities of human stem cell, it is envisaged that a common theme will be the need to expand a large starting population of human stem cell as source material. After which the initiation of differentiation would occur through the formation of human embryoid bodies (hEBs). An initial purification step to harvest the early progenitor population and to remove residual human stem cell will be required. At the next stage, some progenitor cell, for example the neuronal progenitors would be grown on substrates. A second stage of purification of progenitors will be required to remove contaminating cell types ensuring a relatively pure population of cell devoid of other lineages. This would be followed by further expansion of the final phenotype such as cardiomyocytes and dopaminergic neurons on substrate with a separate cocktail of growth factors or feeder cell. A final purification step may be required

where ideally one would want to have only the functional cell type. A schematic of possible processes for three different cell types is shown in Fig. 23.

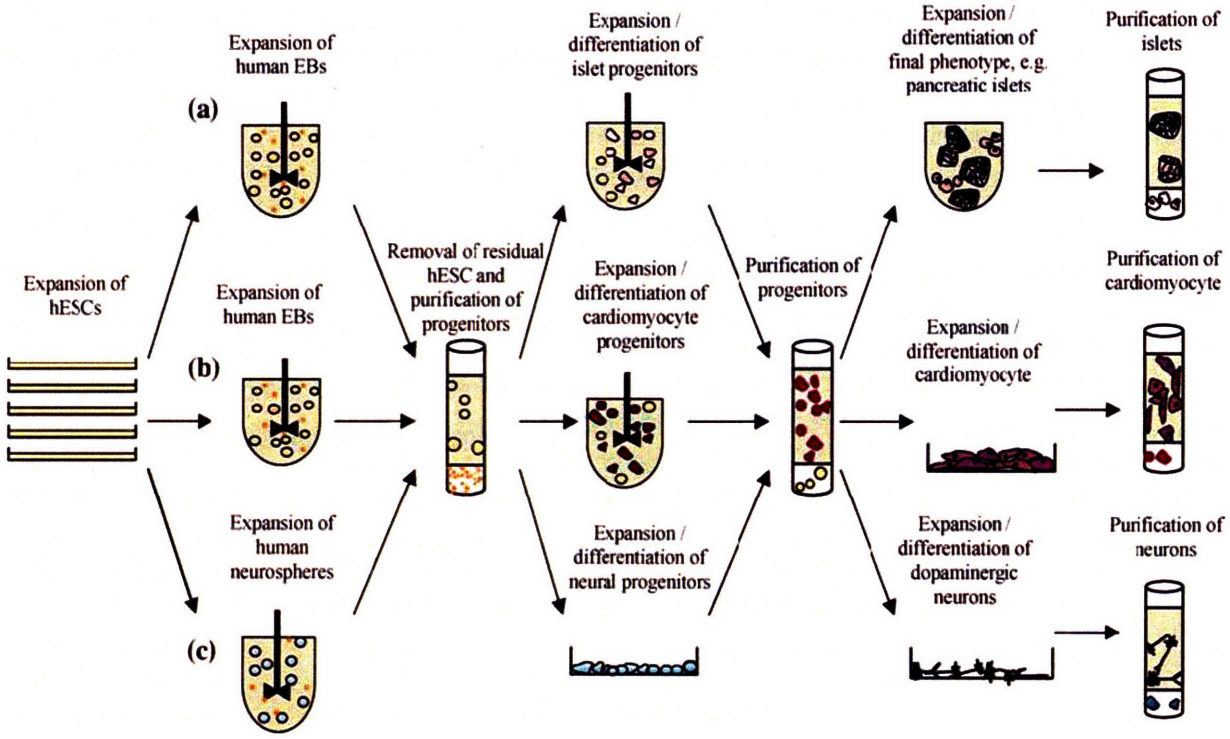


Figure 23: A schematic of possible future manufacturing systems in the expansion and differentiation of various lineages from hESC. The process would begin with generating large quantities of hESC which have an indefinite capability to expand. These resulting cells could be initiated to differentiate in suspension cultures as human embryoid bodies (hEBs) for a period of time. The hEBs would be separated to remove residual hESC and the target progenitor population selected for expansion. Then depending on whether the cell type required grows in suspension (e.g. islets) these would be grown in bioreactors (a), or if they require a combination of suspension culture and anchorage to a surface (e.g. cardiomyocytes), they would be grown accordingly (b). If the neural lineage is required, neurospheres could be cultured, undergo a separation step of enrichment for neural progenitors and eventually specific dopaminergic neurons grown on plastic culture surfaces (c). At the end of the expansion processes, ideally only the final phenotype is purified (49).

Matrices with suitable stiffness are expected to have the clearest influence toward stem cell differentiation during the 1st stage of the manufacturing flow; since the technology of using matrix elastic modulus to direct stem cell differentiation is based on the passive, initial response of stem cell to the microenvironment which is normally expected from a multipotent stem cell awaiting instruction. In addition to that, the matrices with "corrected" stiffness also appear to be able to assist and facilitate the corresponding cell lineages growth and further commitment. Hence, the technology can be applied throughout the whole manufacturing process.

b. Choosing right materials for the elastic modulus defined substrate

It is important to choose suitable materials that result matrices with the "right" stiffness range. Estimates of cellular stiffness derived from different types of microrheological methods cover a wide range depending on the methods used, the type and extent of deformation, and the time that the deformation is applied, as shown in Table 3. The stiffness measured for isolated cultured cell ranges from 0.1 kPa to an approximate upper limit of near 40 kPa (56) for myocytes, similar to the range of stiffness measured for many soft tissues (57). The magnitudes of these moduli can help determine which materials are suitable to construct cell culturing substrate to generate that cellular stiffness.

Table 3: Comparison of elastic moduli measured for single cell in culture (58)

Cell type	Elastic modulus (kPa)	Method
Rat aortic smooth muscle	1.5–11	Elongation between plates
Endothelial	1.5–5.6	AFM
Aortic endothelial Normal/ cholesterol depleted	0.32/0.54	Microaspiration
Endothelial	0.5 cytoplasm 5 nucleus	Uniaxial compression
Inner hair cell	0.3	AFM
Outer hair cell	2–3.7	AFM
Cardiac myocytes	35–42	AFM
Fibroblast	0.6–1.6	AFM
Fibroblast	1–10 (differential stretch modulus)	Uniaxial stretching/compression
Bovine articular chondrocytes	1.1–8	Creep cytoindentation apparatus
Chondrocytes, Endothelial	0.5	Microaspiration
Neutrophils passive/activated	0.38/0.8	AFM
C2C12 myoblasts	2	Cell loading device (global compression)
Alveolar epithelial	0.1–0.2	Magnetic twisting cytometry
Epithelial normal/cancerous	10–13/0.4 – 1.4	AFM
Osteoblast	1–2	AFM
Fibroblasts Normal/transformed	0.22/0.19; 0.42–0.48/1.0	Optical stretcher
Melanoma	0.3–2.0 frequency dependent	Magnetic twisting rheometry
Kidney epithelial Cell cortex Cell interior	0.16 0.04	Magnetic twisting rheometry Tracer diffusion
3T3 fibroblast before/after shear flow	0.015/ 0.06	Tracer diffusion
C2-7 myogenic	0.66	Uniaxial stretching rheometer

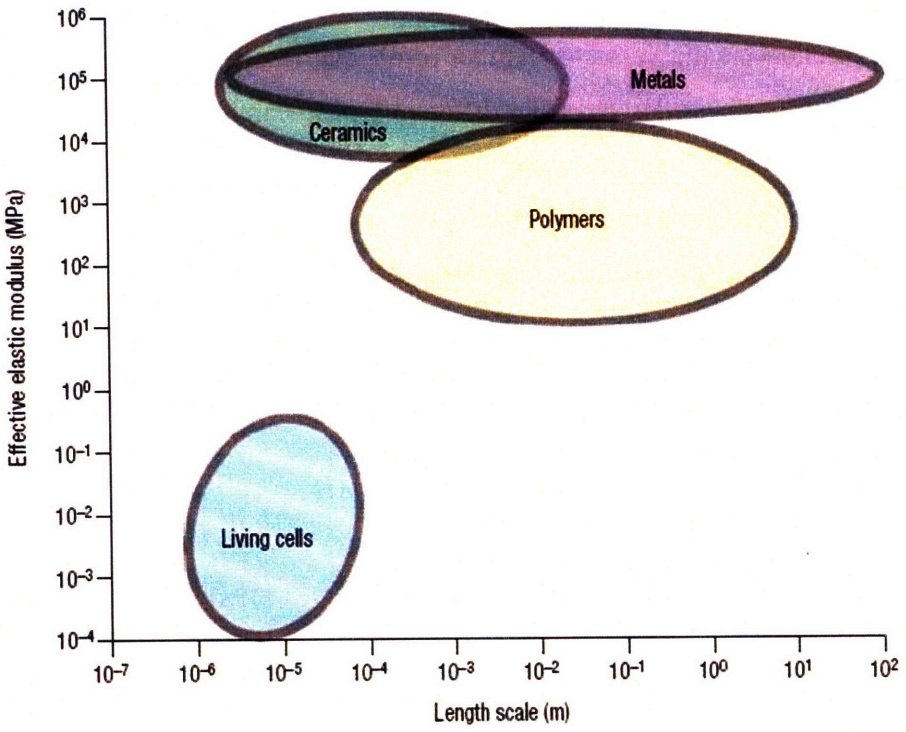


Figure 24: Cell structure and elastic properties, approximate range of values for the elastic modulus of biological cell and comparisons with those of engineering metals,ceramics and polymers (22).

Substrates such as natural or synthetic hydrogels, closely resemble the consistency of soft, native tissues, making them attractive scaffold materials for soft tissue engineering. On the other hand, stem cell' differentiation into connective tissue lineages (i.e. bone, cartilage, ligaments, and tendons) requires materials with higher mechanical strength to closely mimic the tissue mechanical properties. However, hydrogel-like materials can be modified to have increased modulus of elastic modulus, making them more suitable for applications in connective tissue engineering. For example, collagen gels can be adjusted to have a higher modulus by adding HA (Hydroxyapatite), thereby mimicking the composition of bone which is mostly composed of collagen fibers and phosphate minerals. Adding HA to collagen at a 1:1 ratio increases the modulus from 0.392 MPa to 0.422 MPa, which is comparable to trabecular bone (E = 0.443 MPa). Collagen composites can also be designed to contain PLA and chitin fibers to provide increased mechanical integrity and higher human MSC attachment (14).

A silk-based material is also a promising candidate for the matrix with defined stiffness for stem cell culturing. Silk exhibits higher modulus of elastic modulus over other natural materials, such

as collagen. However decontamination and purification methods of silk, prior to their use in vivo, are extremely critical in order to avoid inflammatory and immunogenic reactions. In fact, silk – based scaffolds seeded with hMSCs were shown to induce bone formation in critical-sized, cranial defects (larger than 4 mm) of nude mice, indicated by the presence of bone sialoprotein, osteopontin, and osteocalcin (59). Furthermore, efficient cartilage formation was also seen when differentiating MSCs into the chondrogenic pathway within silk scaffolds (60).

In addition to natural materials, synthetic materials can also be chemically modified to enhance mechanical properties. For example, simply increasing themacromer concentration in photocrosslinked hydrogel scaffolds has been shown to increase the modulus of elastic modulus, such as within (PPE-HA)-acryl Hydrogels (61). A four times increase in the amount of acrylated-PEG reacted with PPE-HA showed an almost 10-fold increase in shear modulus (3 to 26 kPa) (61). Additionally this biodegradable, phosphate-based synthetic material is highly conducive to bone tissue engineering due to the phosphate degradation product, which could aid in overall scaffold mineralization during osteogenesis.

Ideally, synthetic scaffolds with bioactivity may provide physical cues for cell orientation and spreading, which are critical for hESC differentiation and tissue formation. Hence, biosynthetic scaffold with combined natural materials and synthetic materials such as Collagen I coated inert polyacrylamide gels is an appropriate choice since while the synthetic polymer gel provides better substrate stiffness manipulation, the collagen coat will enhance cell attachment and improve biocompatibility of the whole substrate.

In summary, some current technologies in control stem cell differentiation can be used together with the elastic modulus defined substrates and they should be used in that way to enable better control over stem cell' fate. A lot of competing materials are currently in use but each of them is facing their own drawbacks. Combining natural and synthetic materials into a biosynthetic scaffold seems to be a good option to realize substrate with suitable range of elastic modulus for stem cell culture. Industrial requirements for differentiation systems producing cell for vivo usage are extremely stringent and the technology of using elastic modulus stimulus from the substrate alone is not sufficient to satisfy. It is more realistic to aim at the stem cell research and drug screening markets since the cell products in these two markets are for vitro usage and hence, of less stringent requirements.

3. Recommended guidelines for future researches

By reviewing the current stage of the technology and comparing to the current industrial requirements, it is concluded that in order to transform the technology ideas into a successful and reliable commercialized products (matrix for stem cell differentiation and bio assay for drug screening), more research needed to be done and the recommended directions are:

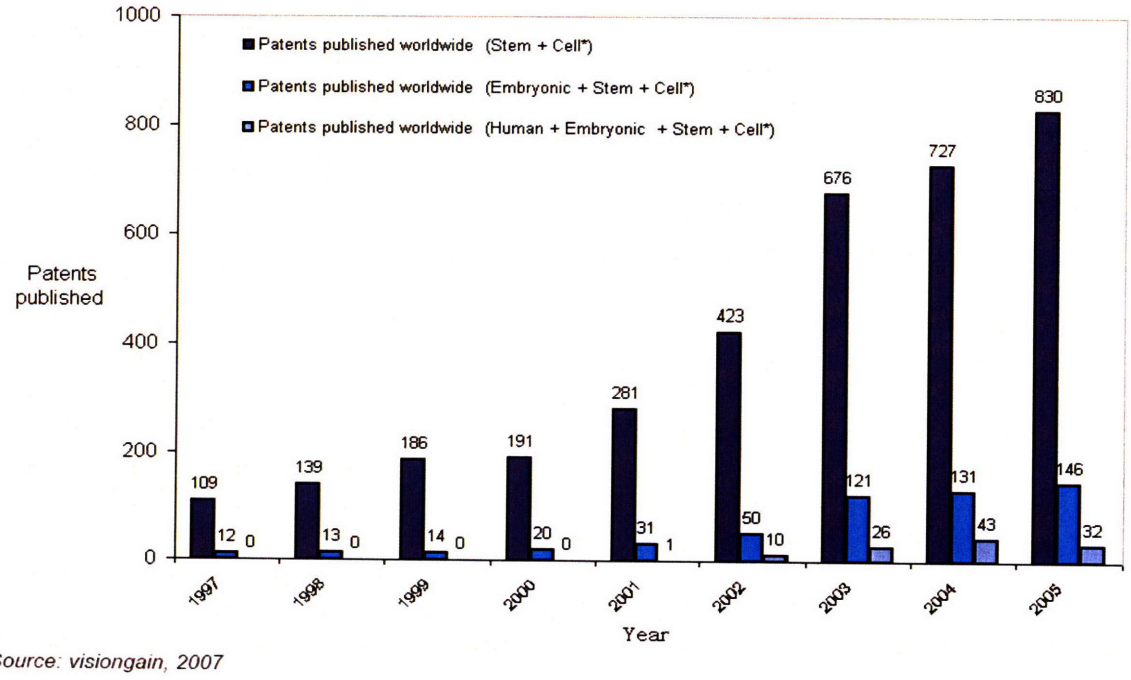
- Determine how sensitive cell are to changes in mechanical properties of their environment.
- Find out how small of a change in elastic modulus that cell can sense.
- Link the expected sensitivity of cell to mechanical cues to requirement for the development of bioassays.
- Choose a suitable material (currently synthetic materials are of good choices).
- Find ways to measure mechanical properties (besides using traditional methods such as AFM).
- Design a microfluidics system to test diffusion and create controlled stiffness gradients differentiation strategies need to be developed for each specialized cell from stem cell.
- Develop propagation strategies for stem cell in vitro.

IV. Patent Analysis

A review of the intellectual properties (IP) are surrounding this new technology was conducted from 2 angles, the first was looking at existing stem cell differentiation methods and induction that are related to matrix and the second was to look at the patent on whole-cell bioassays for pharmaceutical development.

1. Patents related to substrate for stem cell researches

During the years 1980-2005, over 2,000 US patents claiming stem cell technologies and applications relevant to healthcare were published; the annual publication rate accelerated sharply in 2002.



Source: visiongain, 2007

Figure 25: Patent in Stem cell worldwide

The majority of adult stem cell R&D is conducted in the US, while most embryonic stem cell research is conducted outside the US due to a restrictive legislative environment. The chart in Fig. 25 shows stem cell patents distribution among some big companies. As from the chart, companies such as Amgen, Biotransplae are those who hold the most number of patents in stem cell technology.

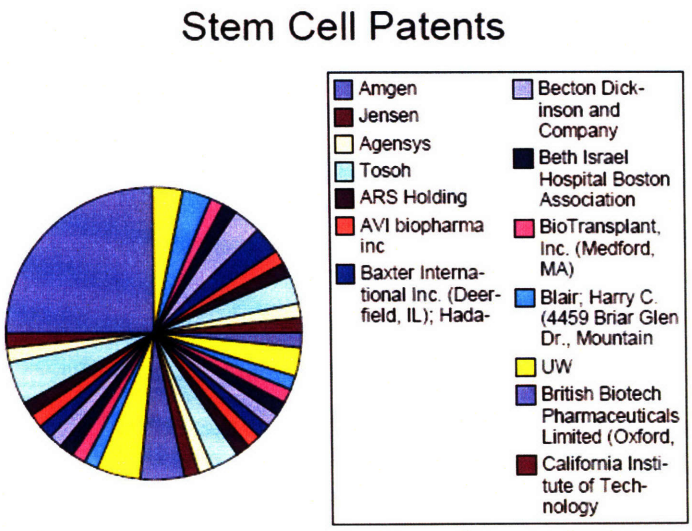


Figure 26: Issued patents including stem cell in the title, Sept. 1, 2005 (62)

Table 4: relevant patents in Stem cell market

US Patent Number **Date Patent Issued** **First Listed Inventor** **Assignees(s)**	**Patent Title**
US2007/0190646 A1 **Aug. 16,2007** **Adam J. Engler,** **Philadelphia, PA**	Regulating Stem Cell Differentiation by controlling matrix elastic modulus
S2005/0058687 A1 **Mar. 17, 2005** **Richard David Guarino et al.,** **Becton Dickinson and Company**	Covalently Attached Collagen VI for cell attachment and proliferation
US007226611B2 **Jun 5, 2007** **Yaizu Suisankagaku Industry Co.,** **Ltd., Shizuoka (JP)**	Glycosaminoglycan/ Collagen Complexes and use thereof

Table 4 listed the three patents that directly related to our technologies: one patents the method of controlling cell differentiation by elastic modulus, one patents the method of controlling cell attachment by collagen VI coating and the last one patents the use of GAG/collagen complexes.

To create new products in light of this current patent environment, we can covalently attach ECM proteins other than collagen VI (e.g., collagen I). The matrix elasticity patent is the most directly applicable, but here we could either license this patent or direct effort toward other cell types such as progenitor cells (downstream from stem cells) or ex vivo tissue cells.

2. Patents related to cell-based assays for drug screening

There are quite a few numbers of related patents to the technology and the assays that we want to develop now. Yet, no one is overlapping and the technology potentially is not going to infringe to any of them.

Table 5: relevant patents in drug screening industry

US Patent Number Date Patent Issued First Listed Inventor Assignees(s)	Patent Title
US 72335353 B2 Jun. 26, 2007 Larry C. Mattheakis et al., Cytokinetics, Inc., South San Francisco, CA (US)	Predicting Hepatocytoxicity Using Cell – Based Assays.
US 20060292695 Dec. 28,2006 Clark AJ, Clark H, Wolf CR, (Midlothian, UK, CXR Biosciences Dundee, UK)	Methods and kits for drug screening and toxicity testing using promoter-reporter cells derived from embryonic stem cells.
US 20060292694 Dec. 28,2006 Clark AJ, Clark H, Wolf CR, (Midlothian, UK, CXR Biosciencs Dundee, UK)	A cell population differentiated from human embryonic stem cells, comprising genetically altered cells so that a promoter that responds to a metabolic or toxicologic change in the cell controls expression of a reporter gene.
US 20060275840 Dec. 7, 2006 Ismail RA, O'Beirne GB, Thomas N GE Healthcare (Chalfont St. Giles, UK)	A solid support for cell-based assays comprising particles including a matrix and having a scintillant substance that has been coated onto or integrated into the matrix of the particles, and adapted for cell growth.

V. Market Analysis

1. Stem Cell Research market

The market for stem cell products and services (including cytokines) is forecast to grow almost three-fold from $24.6 billion in 2005 to $68.9 billion in 2010.

Navigant analysts report that the U.S. and Europe dominate the cell culture market, accounting for 58% and 24% of 2005 revenues, respectively. Growth in the US market is closely linked with increased funding for stem cell research growth, in addition to the expanding set of biologic drugs, the number of which will grow by 20% every year, according to research on the mammalian cell culture market published by Drug & Market Development. The estimated growth of 10 products annually will also mean an increase in biomanufacturing, which depend heavily on cell culture products and mammalian cell cultures.

a. Market Trench

An increasing number of companies have begun to supply reagents and consumables for the isolation, expansion and storage of stem cell and there are immediate trade opportunities in this area. In 2005, stem cell research accounted for $820 million in expenditures with an estimated CAGR of 8.97%, spending on stem cell research should reach $1.26 billion in 2010 and $1.95 billion in 2015, when 20% of the budget will be spent on media and consumable supplies. The current world market for all media and reagent cell culture has been estimated at US$950 million, rising to US$1.8 billion by 2012 as research in this field expands. Such pursuits will be a major factor in the growth of the overall cell culture market, which includes media, serum and reagents. In 2005, the worldwide market generated revenues of $1.02 billion, according to Navigant Consulting. With a compound annual growth rate of 12.7%, revenues should reach $1.86 billion by 2010. The U.S. market alone brought in revenues of $587 million in 2005. By 2010, revenues should reach **$1.2 billion** with a CAGR of 15.6%. Analysts at the Business Communications Company, however, estimate that the cell culture market could generate revenues of **$1.7 billion by 2008**. It is also noted that Market value of all public stem cell companies: $1.655 billion. Currently, each stem cell product is typically assayed by its supplier.

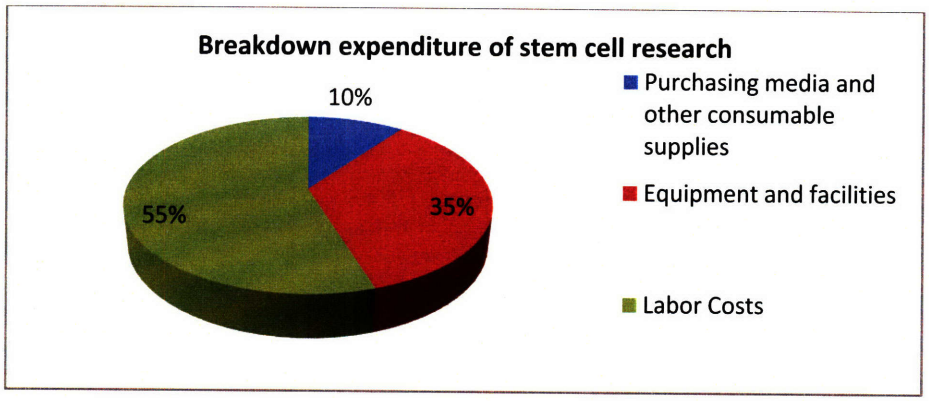


Figure 27: Expenditure of stem cell research

The current global market value for stem cell culture media is ill-defined but is expected to grow as R&D involving stem cell increases. The methodology for the efficient storage with robust recovery and expansion of human stem cell lines, particularly ES lines, is currently also a limiting factor in the widespread adoption of stem cell applications.

Table 6 shows the comparison of the international stem cell factors: only 2 main regions are chosen: USA and some selected countries in Asia. As from the table, USA and Singapore are the 2 countries which have strong and increasing market for stem cell research and stem cell products' commercialization. Both of these countries also have strong funding on stem cell research. For Singapore, this funding comes mostly from the government or A Star Institutes; smaller additional amount comes from universities such as NUS, NTU, and from venture capital.

Table 6: Comparison of the International stem cell sector (63)

Country	Legislation			Funding	Academic strengths	Commercial	Other comments
	SCNT	h ES	ASC				
US	Varies between states. Federal restrictions on hES work			**Strong. California: $1bn / 10 years** Proposed investments **Illinois: $1bn New Jersey: $0.4bn Wisconsin $0.35 bn Mass: $0.1bn**	Lagging on ES work because of federal restrictions but regions of excellence (Boston, Wisconsin), and leading ASC work.	**Increasing. Geron, ACT, Stem Cells Inc and over 20 spin-outs.**	Federal restrictions have limited research, but changing environment will pose real threat to UK.
ASIA							
China	Y	Y	Y	Strong support from Government and regions, although no figures available. Estimated to be largest stem cell programme in Asia.	**Main strength: clinical translation eg Zhu's work.**	Less commercial development.	Some concerns about enforcing legislation, quality standards, cultural differences
Singapore	Y	Y	Y	**Priority area for investment. Significant funding. $7.3m per year by A*STAR committed, plus additional funding from universities, venture capital, JDRF. New $3m funding programme to support stem cell projects.**	National University of Singapore – Dr Ariff Bongso; Dr Alan Coleman, Dr David Lane.	**Strong emphasis. ES Cell International.**	Keen to attract foreign academics and biotech companies
South Korea	Y	Y	Y	**Government priority area. $14m per year to Seoul University, $7.5m per year to Stem Cell Research Centre.**	**World-class expertise in SCNT: Dr Woo-Suk Hwang and Dr Sin Yong Moon, Seoul National University.**	More cautious.	**Opportunities for international collaboration.**

b. Main Players

Currently there are about more than 200 companies dealing with stem cell product worldwide. Specifically for cell culture product market, the market share distribution is as shown in Fig. 28. Invitrogen led the overall cell culture market, generating 34% of revenues. Fisher Scientific, JRH Biosciences, Serologicals, Cambrex and Sigma- Aldrich follow, generating 20%, 14%, 9%, 7% and 6% of the general cell culture market, respectively. As with the general cell culture market in 2005, Invitrogen is also the biggest supplier of stem cell research products. Stem Cell Technologies and Specialty Media, both of which offer specialty niche products, are chief competitors. Other companies are also tapping into the lucrative potential of the stem cell culture market by completing a series of recent acquisitions.

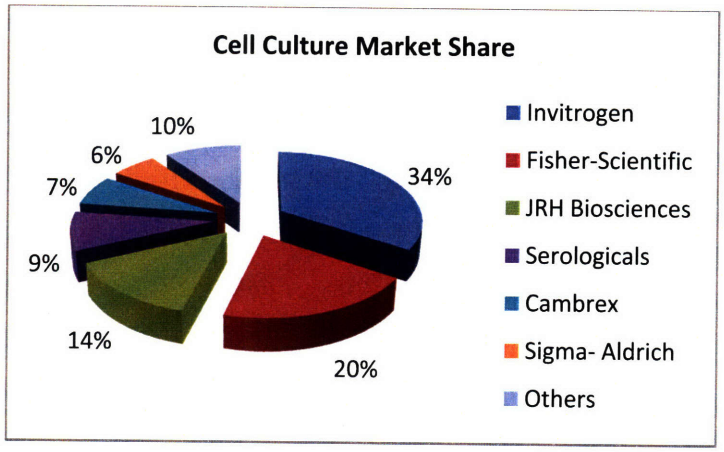


Figure 28: Cell culture Market Share (Navigant Analysis (2005)).

Table 7: Main players in stem cell industry in USA (63)

Company	Description	Financial Information
Geron	Developing cell-based therapeutics based on differentiated hESCs. Proprietary methods to grow, maintain and scale-up undifferentiated hESCs and differentiate them into therapeutically relevant cells including neural cells, cardiomyocytes, pancreatic islet β-cells, osteoblasts, chondrocytes and HSCS.	Market cap: **$398.6m** R&D costs: 2004: $30.1m 2003: $25.6m 2002: $29.8m
Advanced Cell Technologies (ACT)	ACT is the only publicly traded company focusing solely on hES cells. Currently at preclinical stage with work on neurodegenerative disorders, retinal disease and vascular disorders.	Market cap: **$67.3m**
NovoCell	Early-stage developer of cell-based therapeutic products, initially involving islet cell transplantation for treatment of diabetes.	Investments by: BD Ventures Johnson & Johnson
VistaGen	Using hES screening assays to discover drugs for CNS disorders and diabetes	Private NIH grants
Aastrom Biosciences	Developing patient-specific products for the repair or regeneration of human tissues, using proprietary adult stem cell technology Replicell System: unique automated cell production platform used to culture cells	Market cap: **$264.6m**
Stem Cells Inc	Discovery and development of stem cell therapeutics to treat damage to or degeneration of the CNS, liver and pancreas.	Market cap: **$233.8m** Total Revenue: 2004: $0.14m

While there are quite a few of main players in USA stem cell market, these are only 2 main players in Singapore stem cell market: ES cell international and Promatrix (summarized in Table 8). Both of them are heavily funded by the government.

ESI moved entirely to Biopolis in 2004. The majority of its financing has come from the Singapore government, directly or indirectly. Learning from ES history, we see that according to

Colman, who is currently on the company's board: ''the company would have been dead in the water in the US or UK, because for a private stem cell company, it's very tough to raise money,''. It's hard for a stem cell company to be profitable within the time frame that investors would like to see a return.ESI is downsizing its R&D in cell therapies for heart disease and diabetes and upsizing ''more near market opportunities'' like making and selling cardiac cells for drug toxicity studies. From this, we can tailor our matrix for handling cardiac cells to target ESI Company.

Promatrix Biosciences Pte Ltd is the most relevant company to us. It focuses on novel biofunctional and three-dimensional scaffold and related technologies for use in cell therapy and tissue engineering. Promatrix's innovations are licensed from Johns Hopkins Singapore, the international subsidiary of Johns Hopkins University, USA. These technologies enable products that improve ex vivo cell culture outcomes or tissue regeneration in combination with appropriate cell.

Table 8: Main players in stem cell industry in Singapore (63)

Company	Description	Financial Information
ES Cell International (ESI)	Harnessing the potential of hES cells. - Therapeutic focus: developing hES-derived islet cells for brittle diabetes, and cardiomyocytes for chronic heart failure. - Technology platform: focus on scale-up and expansion of clinically applicable hES cells. Also identifying genes and growth factors to grow populations of differentiated cells for therapies. Have developed 6 hES lines, approved by NIH Registry. Collaboration with Hubrecht University, Netherlands for GLP facilities. Supply hES cells, associated reagents and training worldwide. - Aggressive IP policy: Commercial licence agreement from WARF to distribute hES cell lines worldwide. Own IP governing hES cells and culturing techniques, developed in collaboration with Monash, Australia, National University of Singapore, Hadassah Medical Organisation in Israel, and the Hubrecht Laboratory in Netherlands.	Private Funding in 2000: SG$17m Main investors: Life Science Investments Pte Ltd, and ES Cell Australia Ltd (Australian investment consortium). Cash burn: SG$6m
Promatrix	Developing cell expansion and tissue engineering technologies, based on the expansion of HSC cells in scaffolds. Spun out of John Hopkins, and based at the BioVenture Centre Singapore, a joint venture between Becton Dickinson & Co and John Hopkins Singapore	Shareholders: Biomedical Science Investment Fund BD Technologies BioVenture Centre

c. The Targeted Market – Stem Cell Research in Singapore

Table 9 provides a glance at the stem cell industry environment in Singapore. The government has established a $600 million fund to invest in startups engaged in research on stem cell and other cutting-edge life-sciences projects. Singapore opened Biopolis, a 2 million-square-foot complex of laboratories and offices devoted to such research. So far, Singapore has pointed up $22 million for ES Cell International. ES today owns six stem-cell lines (a line is a group of identical cell that come from the same embryo) and is focusing on developing treatments for diabetes. Clearly, "the center of excellence in stem cell research" created will greatly facilitate the development of stiffness-defined matrices which has direct applications in stem cell handling and culturing.

Table 9: Stem Cell Industry Environment in Singapore (63)

Regulatory framework	The Human Cloning and Other Prohibited Practices Act was passed in 2004, allowing SCNT while prohibiting reproductive cloning, the import or export of cloned embryos, and commercial trading of human embryos, eggs or sperm. The full regulatory system is not yet in place, but includes plans to establish a statutory authority to issue licences for research on human embryos.
Government support / funding	Stem cell research has been identified as a priority area for investment in Singapore, and is one of the cornerstones of the $2 billion National Biomedical Science Strategy announced in June 2000. A*STAR (Singapore's Agency for Science, Technology and Research) funds $7.3million per year to support stem cell research, with additional funding from universities, venture capital, JDRF and other funding bodies. The emphasis is on knowledge transfer to drive industry growth, and infrastructure is strong, including shared facilities at the Biopolis hub.
Research clusters	- National University of Singapore 12 groups working on stem cells, including Ariff Bongso, a leading expert who collaborates closely with Monash and Hebrew Universities. Other research centres: - Institute of Molecular and Cell Biology (IMCB) - Bioprocessing Technology Institute (BTI) - Genome Institute of Singapore (GIS) - Singapore General Hospital and National Cancer Centre There is currently a skills gap with well-trained researchers.
Companies	- ES Cell International (ESI) - Promatrix - Cell Research Corporation - CyGenics

2. Drug screening market

The pharmaceutical industry is under constant pressure to improve efficiency across the drug development process. One of the bottlenecks is the screening of potential drug candidates, using biological assays to demonstrate efficacy. Currently, cell from animals or atypical tissue are used, but there is a difficult balance between clinical relevance and screening capacity. Human ESC derived cell lines offer a real alternative, with a homogeneous and high density array of a disease-specific cell in a format suitable for screening. This could dramatically increase efficiency and decrease use of animals.

a. Market Trench

The market is dealing with an important phase in drug development, which is screening and eliminating potential drug candidates that manifest toxic properties in vivo. In Fig. 29, ADME stands for **Absorption, distribution, metabolism, excretion, and toxicity tests** which are normally used to characterize a compound's properties with respect to absorption by the intestine, distribution to the organism, metabolism by the liver, excretion by the kidney, and toxicity profiles. And this phase take center stage given the large fraction of lead compound and drug failures associated with toxicity properties.

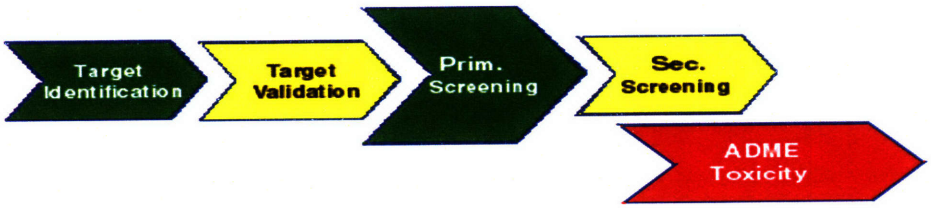


Figure 29: Phases of drug development (64)

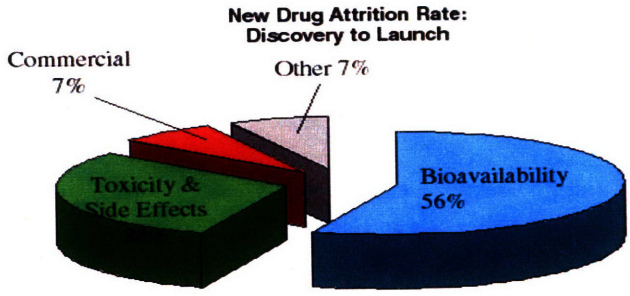


Figure 30: New drug Attrition rate, from discovery to launch (64)

As illustrated in this Fig. 30 Thirty percent of the total new drug attrition in the developmental pipeline is attributed to toxicity profiles and side effects. Hence, this is an area that the

pharmaceutical companies and other drug discovery and development entities are paying close attention to. And this portion of the pie is the one that we are aiming at.

Quantitatively, in terms of the quantitative market opportunity in the ADME and toxicity screening space, almost $3 billion was spent on various ADME and toxicity studies in 2001. Note that this figure includes the hugely-expensive animal studies that take place late in the process of drug development, which are not affected by the upstream migration of ADME and toxicity screening, and overlapping into the screening (primary and secondary) space, as a result of growth in drug targets and hence their interrogation. Fig. 31 presents a breakout of how this total (quantitative) market opportunity segregates into its individual components. It is noted that $0.20 billion is spent for in vitro toxicology, which is including the expecting expenditure portion that drug companies spend to purchase our matrixes.

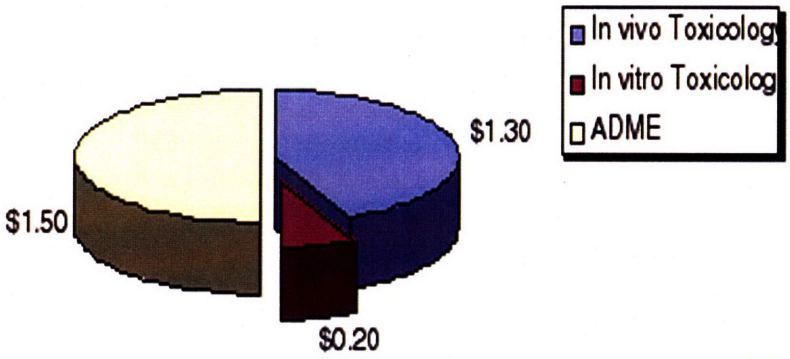


Figure 31: ADME/Tox Screening Market ($, Billions) (64)

All candidate drugs must be screened to ensure safety. However animal models are expensive and low-throughput; 16% of drugs currently fail in Phase III trials because of adverse effects in man(65). Introducing in vitro tests could improve this but sourcing human liver cell (hepatocytes) is difficult. Currently, primary human hepatocytes are obtained from cadavers or from patients after operations, but the supply are limited, of variable quality, and difficult to maintain in culture. Stem cell could provide a source of specialised hepatocytes for toxicity testing and ADME screening.

Table 10: Biological Assay systems for the Evaluation of Cytochrome P450 Enzyme Isoforms (66)

What is measured?	Source of the enzyme(s)	Availability	Advantages	Disadvantages
Selected cytochrome P450 enzymes	cDNAs that express the enzyme	Readily	Adaptable to high-throughput screening. Can study single cytochrome P450's at a time.	One drug metabolizing enzyme at a time is assayed.
Selected cytochrome P450 enzymes	Immortalized cell lines expressing the enzymes	Readily	Nonlimited source of enzymes.	One or a few drug-metabolizing enzymes can be studied at a time.
Cytochrome P450 enzymes	Microsomes	Good	Easy to obtain. Commercially available. Relatively inexpensive	Only Phase I drug-metabolizing enzymes can be addressed.
Some cytochrome P450 isoforms	HepG2 cells (a cell line)	Readily	Some drug-metabolizing enzymes can be interrogated. Long-term function can be studied.	Limited interrogation of Phase I and Phase II drug-metabolizing enzymes.
Some cytochrome P450 isoforms	Primary hepatocytes	Poor	Most drug-metabolizing enzymes and cytochrome P450 induction studies can be performed.	Some Phase I and Phase II drug-metabolizing enzymes decrease over time.
All cytochrome P450 enzymes	Liver slices	Poor	All drug-metabolizing enzymes can be studied. Cell-cell contacts can be studied.	All Phase I and Phase II drug-metabolizing enzymes decrease over time.
Some cytochrome P450 enzymes	Liver spheroids	Poor	Most drug-metabolizing enzymes can be studied. Some cell-cell contacts can be studied.	Some Phase I and Phase II drug-metabolizing enzymes can be studied. Must be made fresh.
All cytochrome P450 enzymes	Rodents and dogs	Readily	All drug-metabolizing enzymes can be studied, but the biological systems are not human.	All Phase I and Phase II drug-metabolizing enzymes can be studied, but these are of animal origin and not of human origin.

Table 10 provides a snapshot of the various biological assay systems for ADME and toxicity screening. Given the central and essential role of the liver in processing out toxic compounds from the system, the pharmaceutical industry is interested in having proxies of the liver for the purpose of screening compounds against in their quest to "screen out" hit compounds with problematic ADME and toxicity profiles. For this reason, hepatocytes have attracted a significant share of the marketplace for ADME and toxicity screening, and Table 11 presents different hepatocyte assay systems and their respective value drivers (in this manner, we explore the

landscape of approaches and technologies that the industry is deploying to address the fundamental ADME/Tox properties of compounds). In the above table, model systems and approaches that are predictive of human toxicity are highlighted. This is perhaps the most crucial element of an ADME and toxicity screen – how strongly predictive is the assay approach. Generally, human hepatocytes are highly predictive in this setting, as they are the "closest proxy" to the *in vivo* situation. However, their availability and reproducibility of the conditions of the experiment are critical bottlenecks in the process, and hence, the industry continues to search for technologies that are predictive, robust (reproducible), and cost-effective. And this gap in the market naturally opens up chances for our matrices since we can provide a well-controlled way to manipulate and culture liver cell.

Table 11: Hepatocyte Assay Systems (66)

Model System	Tissue-specific function	Duration of function	Predictive of human toxicity	Reproducibility	Availability
HepG2 cell line	-	++	-	-	+++
Monolayer hepatocytes (rodent)	-	-	-	-	+++
Monolayer hepatocytes (human)	-	-	+	-	-
Suspended normal hepatocytes (rodent)	-	-	-	-	+++
Suspended normal hepatocytes (human)	--	-	++	-	-
Liver microsomes	+	+	-	-	-
Tissue slices (rodent, dog, primate)	++	-	-	-	-
Tissue slices (human)	++	-	++	-	-
Rodents	+++	+++	-	+	+++

The cell based screening market is estimated to be worth US$700million per year by 2009; pharmaceutical companies are increasingly interested in the use of stem cell lines as drug discovery tools (67).

Looking into the culture of the market, it is noted that the field of high-throughput screening has created several types of strategic alliances (65). The technology access agreement is a common way whereby a large company gets to use a new technology and participate in its late-stage development. For that privilege, the pre-commercial stage partner tries to obtain up-front payments, research and development payments, milestone payments, and royalty payments for resulting products.

The overall pattern for the industry serving pharmaceutical high-throughput screening is one of steady growth at rates exceeding pharmaceutical sales growth rates by a considerable margin. Growth rates for high-throughput screening exceed even rates of growth for pharmaceutical research and development. Even these high growth rates for screening lag behind the actual needs of the industry. Growth in high-throughput screening product and service revenues will be constrained by caps placed on pharmaceutical research and development expenditures in order to maintain acceptable profit margins (65). The revenue growth rates reflect a balance between burgeoning opportunity and the realities of spending constraints. The pharmaceutical industry is challenged to increase its output of innovative new drugs while maintaining profit levels that are acceptable to the investment community.

Therefore, the future of high-throughput screening will be determined to a large extent by the level of funding committed to that activity by pharmaceutical companies. The levels of funding will be determined by a complex equation for optimization of the entire drug discovery process. A key element in such equation is that the discovery task has shifted from simply identifying promising leads to the added proviso that dead–end leads should be eliminated from consideration as early in the process as possible. Some inputs to the decision equation are: value received for miniaturization versus resource inputs required; the extent to which new technology provides value that extends beyond the primary screening process; the information content provided by new technology; and the technology's "homogeneity index"(65). Whether a new technology or instrument is adopted will also depend on the extent to which it provides

laboratory integration without destroying flexibility. Pharmaceutical companies are recognizing that their future success is tied to viewing the entire drug discovery process as a single entity.

In summary, it appears that opportunity exists for great diversity in high-throughput screening technologies and systems. The dominant theme, continual evolution, is driven by economic realities and pulled along by ever-increasing requirements for increased information content. Diversity and flexibility appear necessary, and computers will make order out of the chaos and permit creative new ways of viewing and mining data. Information systems can go far toward integrating the entire drug discovery and development process (65).

b. Main Players

CXR Biosciences, based in Dundee, is collaborating with Geron and the Roslin Institute to develop and commercialise hESC-derived hepatocytes for *in vitro* assays. Geron will contribute hES cell lines and IP, CXR bring expertise in hepatocyte screening models. It is expected that Geron will produce cell lines to sell as kits to pharmaceutical companies, while CXR will offer contract screening services to smaller companies without in-house facilities. They will be our direct competitors.

We can consider Affimatrix as an example of expected financial status: in the first quarter of this year, 2008, the company reported a product avenue of $62.8 million, in which $58.8 million comes from bio array and stem cell agents. This again confirms a big available market about whole cell based for high throughput bio assay.

c. The Targeted market – Drug Screening Market in Singapore

Singapore is emerging as a growing base for drug discovery and development activities. In particular, the biopolis has attracted many pharmaceutical and biotechnology companies to establish R&D operations in Singapore. These companies include AstraZeneca, Bristol-Myers Squibb,Covance, Eli Lilly, GSK, Icon Clinical Research,Merck and Co.,Novartis,Novo Nordisk, Pfizer, PPD, Quintiles, Sanofi-Aventis and Schering-Plough. Singapore also continued to strengthen its position as a strategic manufacturing base for global pharmaceutical companies: Novartis Pharma AG started construction of a new production facility to provide needed capacity within its global manufacturing network. In addition, GSK completed an expansion to its existing manufacturing facility in Singapore, while Pfizer opened a multi-purpose facility, making Singapore home to its first large-scale manufacturing plant in Asia.

With this growing trench and encouraging policy from government, Singapore is in a good position to capitalize on the interest of whole cell-based assay for drug screening, but new potential products must be introduced quickly into the markets to gain first mover advantage, and to capture the value of this market. The stiffness defined extracellular matrix must be able to provide convincing evidences that it is a robust solution for drug screening and to demonstrate the competitive strengths of the technology compared to other impressive assays.

A note of caution is also necessary. It may not be easy to sell cell lines to large pharmaceutical companies. It could take a long time to validate the assays, the technology is not proprietary, in some instances the IP position is unclear, and the research costs are extremely high. Pharmaceutical companies may therefore be wary to engage in deals with biotech companies or, if they do see potential in the technology, may prefer to develop expertise in-house. A model of collaborative development with academia may be more appropriate. This is likely to be slow-growing as a commercial opportunity.

VI. Cost Model

In this section, an estimated fabrication based cost model will be constructed. The cost model is applicable for the startup company which fabricate matrix with controlled elastic modulus for stem cell research and bioassay development. The targeted customers are stem cell researchers and drug screening service providers.

1. Product Description

The matrix is a collagen I-coated polyacrylamide gel with three controlled parameters: elastic modulus E, thickness h and cell adhesion. The matrix is in circular shape with a range of diameters. The matrix is capable of inducing human stem cell (both embryonic stem cell and adult stem cell) into 3 different specific lineages: neural type, muscle type, and bone type with basic culturing media. The preferred cell differentiation can be inhibited by introducing blebbistatin into the ECM. The matrix with blebbistatin introduced will function as normal a collagen coated petri disk. Anchorage dependent cell introduced into the matrix can also be reengineered to develop into different types of cell if a robust and proper culturing media is used.

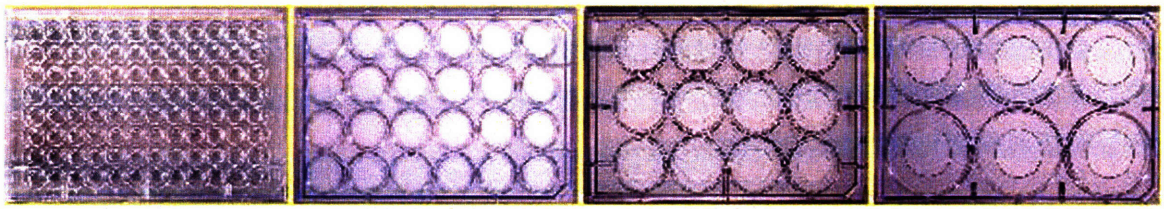


Figure 32: Product Illustration

✓ Product specification:

- Dish diameter/plate type
    - 35 or 50 mm diameter dish
    - 6, 12, 24, and 96 multi-well plates
- Substrate thickness: dependent on desired cell lineage that customers want to differentiate stem cell into
- Coating: Collagen I
- Substrate elastic modulus: dependent on desired cell lineage that customers want to differentiate stem cell into:

- Neural cell: $E_{substrate} = 0.1 - 11$ kPa
- Muscle cell: $E_{substrate} = 8 - 17$ kPa
- Bone cell: $E_{substrate} = 30 - 34$ kPa.

2. Manufacturing Process

Fig. 33 shows a possible manufacturing process for the product. First, polyacrylamide was formed by triggering polymerization of the polymer's precursor. After that, the gel is coated with collagen I. Circular gel disk, each of 30mm in diameter are then cut out into pieces. They are then passing through quality control process where their elastic modulus and thickness as well as collagen concentration on the surface will be measured to meet the specifications. The elastic modulus measurement will be done by nanoindentation test with the aid of an AFM (31). Also, AFM is also used to measure the matrix thickness. The expected yield of the process is 80%. After that, the "pass" gel disk will then be wrapped in sealed air packaged. They are now ready to be shipped to the customers.

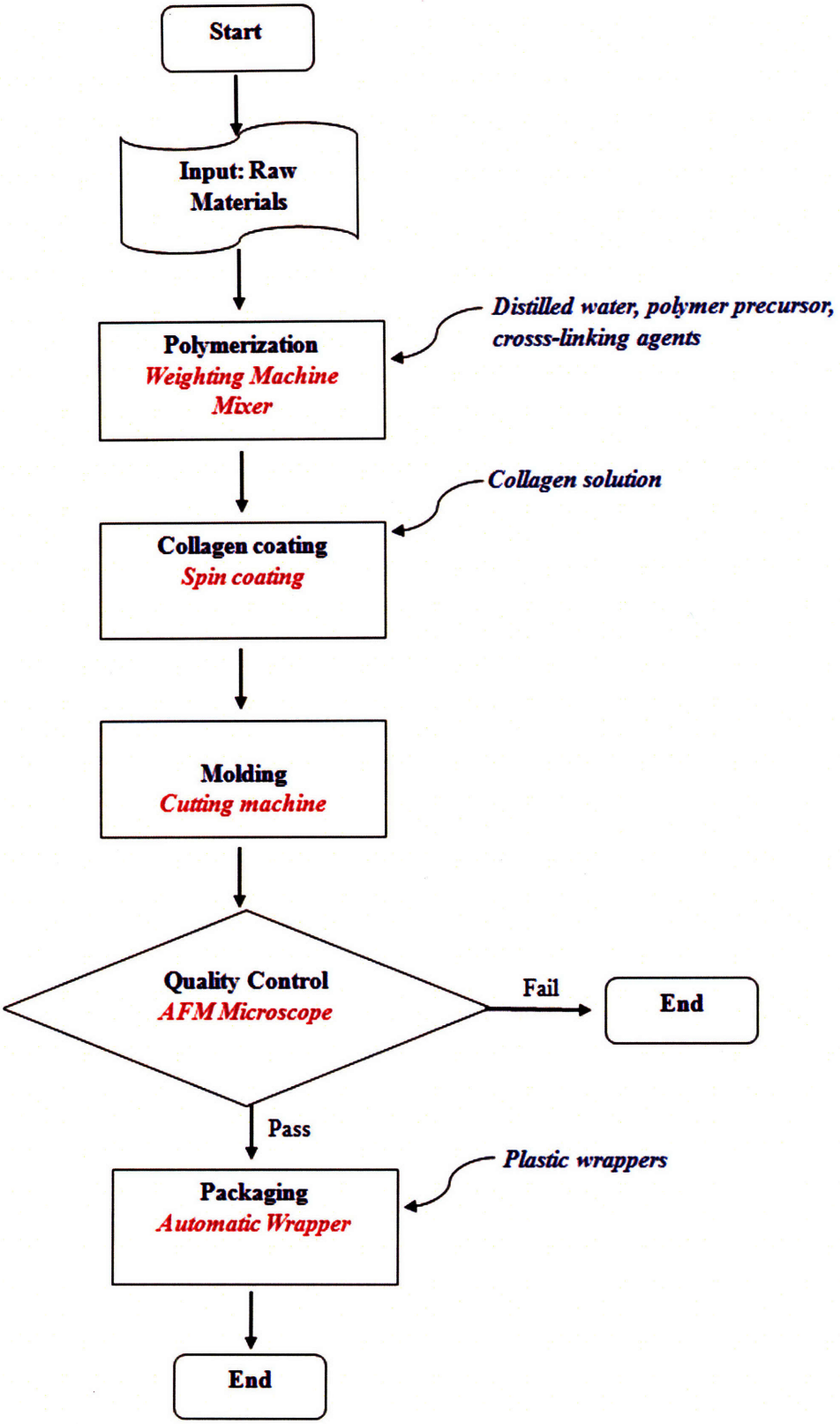


Figure 33: Manufacturing Process Flow Chart

3. Cost Model

The main results of our cost model are tabulated in the following Fig. 34. It is noted that licensing fee for the patent on "regulating stem cell differentiation by controlling matrix elastic modulus (US 2007/0190646)"is not taken into account in this cost model.

Production Capacity	300,000	lots/year
Anuual Production Volume	300,000	lots/year
Lot Size (1m x 1m)	943.6188	dish/Lot

Cost Summary

VARIABLE COST ELEMENTS	$/lot	$/year	%
Material Cost	$ 51.52	$ 15,456,562.50	81.64792392
Labor Cost	$ 11.32	$ 3,396,868.13	17.94365534
Energy Cost	$ 0.26	$ 77,317.10	0.40842074
Total Variable Cost	**$ 63.10**	**$ 18,930,747.72**	

FIXED COST ELEMENTS	$/10 year	$/year	$/lot
Main Machine Cost	$ 410,450.00	$ 41,045.00	$ 0.14
Fixed Overhead Cost	$ 300,178.38	$ 30,017.84	$ 0.10
Building Cost	$ 210,000.00	$ 21,000.00	$ 0.07
Auxiliary Equipment Cost	$ 82,090.00	$ 8,209.00	$ 0.03
Maintenance Cost	$ 155,112.50	$ 15,511.25	$ 0.05
Total Fixed Cost	**$ 1,157,830.88**	**$ 115,783.09**	**$ 0.39**
Total Fabrication Cost		**$ 19,046,530.81**	

Cost Model Output		
Variable cost/unit	$	63.10
Fixed cost/unit	$	0.39
Total Unit Cost	**$**	**63.49**

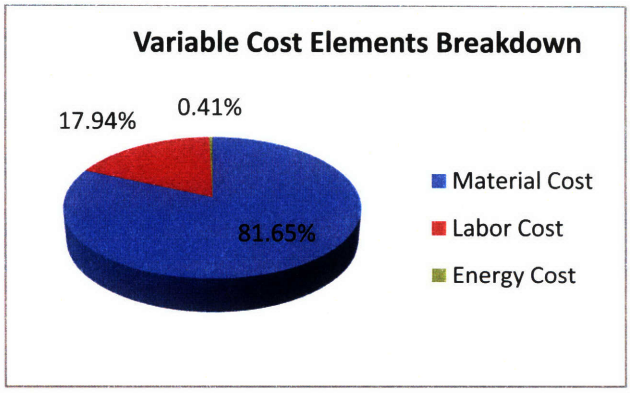


Figure 34: Variable cost breakdown

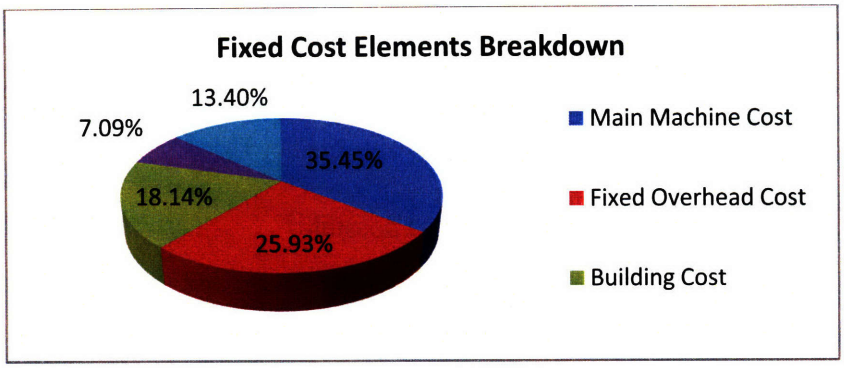


Figure 35: Fixed cost breakdown

Based on the materials and equipment costs as well as other assumptions which are tabulated in details in the Appendix, cost for fabricating a lot of approximately 1000 dish is $63.49. Hence, the cost for each dish is about $0.068/dish.

Our direct competitors are petri dish suppliers with normal collagen coating for cell attachment enhancement. By looking at the market and carrying out small investigation on the current market pricing of culture dishes, we get the price for each petri dish of same size (30 mm in diameter) with similar collagen coating concentration is about $4.80/dish from MatTek Corporation (68). On the other hand, a non coating FluoroDish Sterile Culture Dish (35mm in diameter) costs from $1.50/dish to $3.00/dish from World Precision Instruments company (69).

Hence, we can provide dish with a better price, say 50 times our cost of production (=50*$0.068/dish = $3.40/dish) and a much better control over cell fates. If selling at that price, we are getting a profit of $3.33/dish (499% profit).

Table 12: Profit per unit

	Price – Cost = Profit
An Elastic modulus controlled Collagen I – coated polyacrylamide dish	$3.40 - $.067 = $3.33

VII. Business Analysis

From the inventors' perspective, there are 3 business models that could be used for a novel technology:

- Sell the patent and ideas for the matrix as soon as possible
- Use the patented technology to reproduce the matrix in – house and
- Use the patented technology to produce the matrix but out – source the actual manufacturing.

It can be assumed that all the work completed to discover and develop this novel technology is involved in a long – time fund, meaning that until recently, no venture capitalists or similar entry had financed the project in anyway, meaning, currently, there is no rush to pay back investors. In addition to that, at this point, there are numerous risks related to the elastic modulus controlled matrix because technically, a significant amount of development still needs to be completed before a sellable product is made. Hence, in the inventors' shoes, we will not go with the first option.

For the last two options, a company needs to be started and the exit strategy would be to eventually sell the company. And the company can be sold at any pints of time. As the company moves upper and upper in the supply chain (or closer and closer to the final customers), higher values added into the products and higher profit the company can earn.

1. Risks

The risk in starting a company based on this novel technology can be divided into two categories: technical and market.

a. Technology risks

Since the elastic modulus controlled matrix is only for vitro – usages, less stringent requirements will be needed compared to other in vivo matrices/scaffolds. In fact, the elastic modulus controlled matrix does not need FDA approval. This is a huge advantage since it will save a lot of time, effort and hence, money.

Besides that, there are still certain technical risks that associate with the matrix. The main technical risks are:

- The necessary mechanical properties of the matrix cannot be obtained.

- The probability that the introduced anchorage dependent stem cell can differentiate into the specific cell type that we want (success rate).

In order to develop a sellable product, we need to keep the following issues in mind:

- **Need for further research:** more research needed to quantify matrix's elastic modulus effect to the probability of cell differentiation into specific lineage. Also, best combination of growth media with the range of elastic modulus to maximize the control of cell fate need to be identified.
- **Efficacy:** quantify the matrix's efficacy in set of equations.
- **Scale – up and manufacture:** the most proper manufacturing plan and a detail plan to scale up manufacturing is needed to quickly grasp the market share.
- **Regulations (especially in stem cell):** Stem cell regulations are different from countries to countries. Even inside USA, they are different from regions to regions. Hence, plans to penetrate into each specific market need to account for this carefully.

b. Market risks

i. Stem cell research market

Overall, the stem cell research market is very dynamic. Researchers are always striving for better solutions and methods for stem cell manipulation and applications. Hence, the market is considerably easy to penetrate. Convincing stem cell researchers to use our elastic modulus controlled matrix should be relatively simple. They are eager to try out new things; especially they can see the matrix effect quickly and easily.

Yet, also because of this intrinsic dynamic property of the stem cell market, it is hard to survive in this market with numerous competitions from other newly developed technologies such as using nanograting pattern or hydrogels to induce specific cell differentiation. It is clear that the elastic modulus controlled matrix would hold a number of advantages over current stem cell culture methodology, but many of the newer products also hole these same advantages (efficacy, genome homogeneity, inexpensive, etc).

However, so far no technology has been proven to be capable of control stem cell' fate by itself. Our matrix, in fact, has the potential to achieve that goal. With only varied matrix elastic modulus while the growth media and culture time keeps the same, different cell fates can be induced. This helps our matrix stands out among the rest since it can be combined with any

optimal growth media to induce efficient cell differentiation. Therefore, there is a large are of research that develop new culturing agents, media and growth factors that completely in compliment with our technology. If our product hits the marketplace first and establishes itself, then any technologies requiring a matrix to handle cell can be incorporated together with our matrix and would no longer be competition but a possible enhancement.

Another advantage of our product is that it could be designed and tailored specifically to induce stem cell to differentiate into 3 different cell types. In stem cell research, especially stem cell therapy, there are distinctive specialization areas where stem cell are desirable to differentiate into only certain types of cell for example: kidney construction needs kidney cell, liver construction needs liver cell, Parkinson's study needs neural cell, orthopedic reconstruction needs cartilage and bone cell, etc. Most of the technology can only induce stem cell into a single specific lineage. Therefore, their target market is inherently small, enclosed into specific research areas. In contrast, the elastic modulus controlled matrix's target market is more general. Our technology's approach to stem cell differentiation is from fundamental, which generally apply to most of anchorage dependent cell types, making our market scope is wider.

ii. Drug screening market

In contrast to the stem cell market, the drug screening market is quite conservative. It's more difficult to penetrate into the market. There are high entrance barriers for new technology since the effect of new technology can only be evaluated in a long time range. Therefore, big pharmaceutical companies – our targeted customers are reluctant to switch from their conventional ways to our novel technology. There is a huge need for us to convince them on our matrix's efficacy through both our data and our successful from the stem cell research market.

However, once we are inside this market, there are higher chances for us to be successful than in stem cell research market. First, the drug screening market share is much bigger and less segmented by few big companies like in stem cell market. Second, this market is also of higher growth rate and of higher demand. Once the elastic modulus controlled matrix can gain big pharmaceutical companies' trust, we can survive and stand inside the market for long time without much threat from other novel competing technologies.

In conclusion, there are risks associated with our technology's commercialization for both of our targeted markets. While stem cell market pose less risk technically, drug screening market pose less risk in business-wise. This indicates that the strategy of initially focusing on stem cell research market is of higher probability for success. We are then using the proven success in stem cell research as a positive force to penetrate into the drug screening market.

2. Business Strategies

a. Integrate into the Stem Cell Research Market Supply Chain

Fig. 36 shows the stem cell market's supply chain.

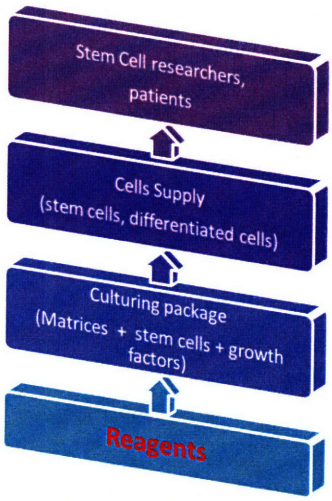


Figure 36: Stem cell industry's supply chain

For stem cell research market, we expect to start at the reagent markets, and slowly penetrate onto the culturing package provider and finally our aim is to supply cell with well – defined morphology and linage. As time goes on, we are move forward, penetrate deeper and deeper into the current market. In the same time, we increase the values of our products and gets higher market share. In the short-term the consumer will mainly be academics and cash-poor stem cell companies, limiting the size of the market. However, in the medium term it is likely that there will be much more value in providing research products. As with genomics, the companies which realized most value were the ones, such as Invitrogen and ABI, selling material to researchers and companies working on gene-based therapies.

b. Integrate into the Drug Screening Market Supply Chain

Fig. 37 shows the drug screening's supply chain, a typical drug development phases and the corresponding company filter. Initially, in the drug development stage: it starts from a large amount of compound, through testing, trials; at the end of the day typically there is only 1 pill of drug that surpasses all the tests. Similar to that is distribution among companies.

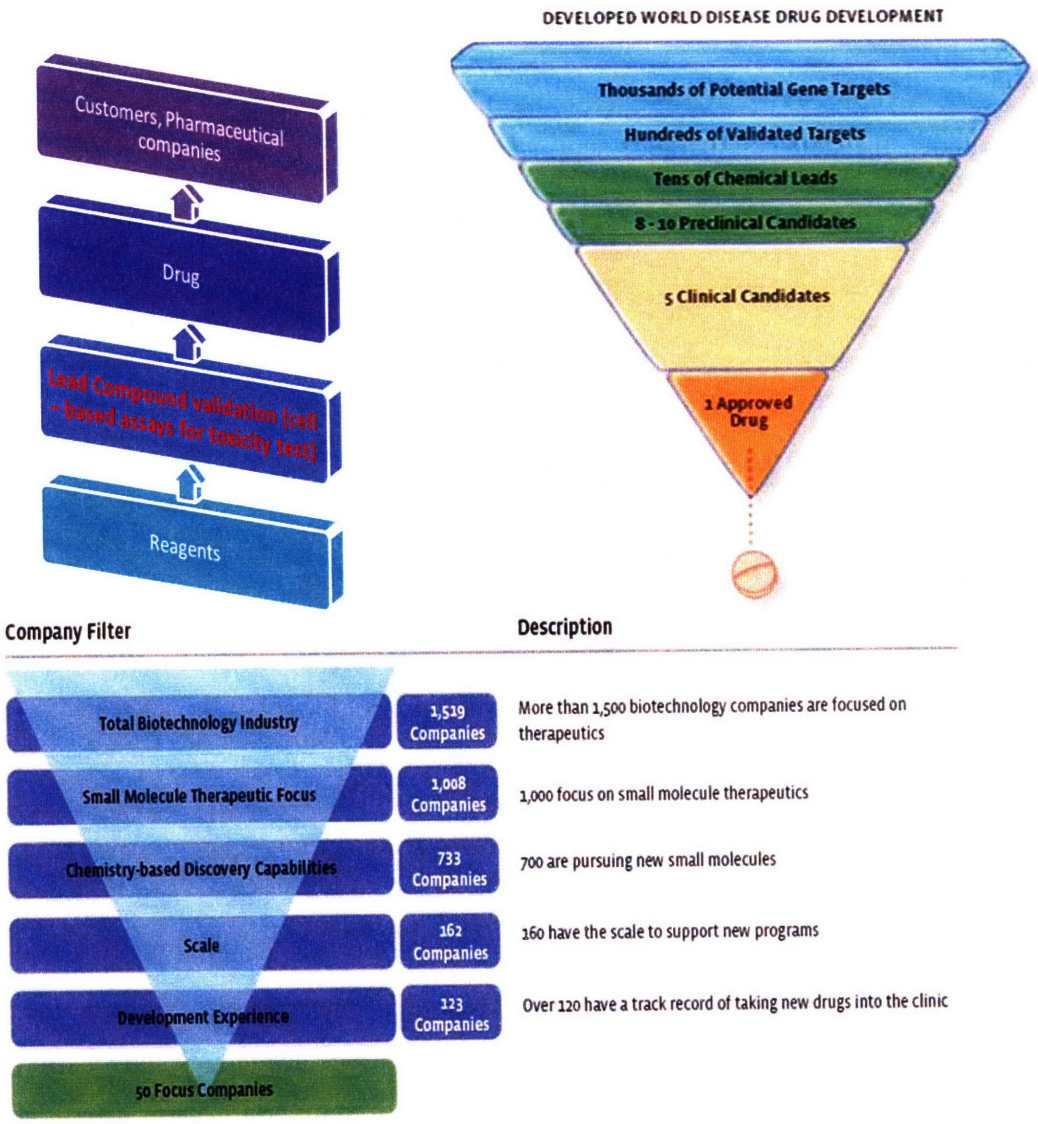


Figure 37: Drug screening industry's supply chain (70)

In the world, there are approximately 1500 biotechnology companies which are focused on therapeutics, 1000 focus on small molecule therapeutics and only 120 have a track record of taking new drugs into the clinic. This indicates that we should also start from the initial stage of

the drug screening spectrum to gain better market share. So for out pharmaceutical supply chain, we aim to position at the lead compound validation center (red in Fig.37). Our long time goal is to position at the drug development company in this supply chain.

c. Summary

Fig. 38 summarizes our approach into both of the targeted markets. The elastic modulus controlled matrix will provide better research tools in manipulate and culturing stem cell. After that, it will incorporate into toxicity testing procedure, and add value to drug screening process. Finally, our long time goal is to fabricating different cell types from stem cell, providing materials and solutions for cell therapies.

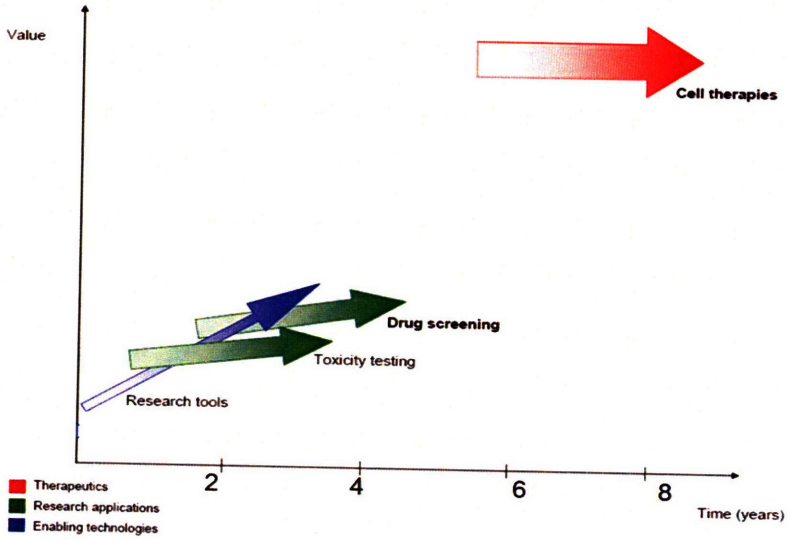


Figure 38: Technology development trench (63)

In a clear time scale, it is a good plan to set up a company this year, gain license for the important patent on elastic modulus controlled matrix for stem cell differentiation. At the early stage of our business, we will synthesize the matrix; sell them to bio researchers and the whole cell bioassay developers. At the intermediate stage, we move on making our own package by combining our matrix with the relevant reagents, as well as develop instrumentation and technology to fabricate our microfluidic miniaturized assay. At the later stage, we aim at being a reliable cell supply first in the regions (Singapore, Asian countries) and then to the USA and worldwide market. Parallel with that, we will provide drug screening and testing methods for contracted pharmaceutical companies in Singapore as well as worldwide.

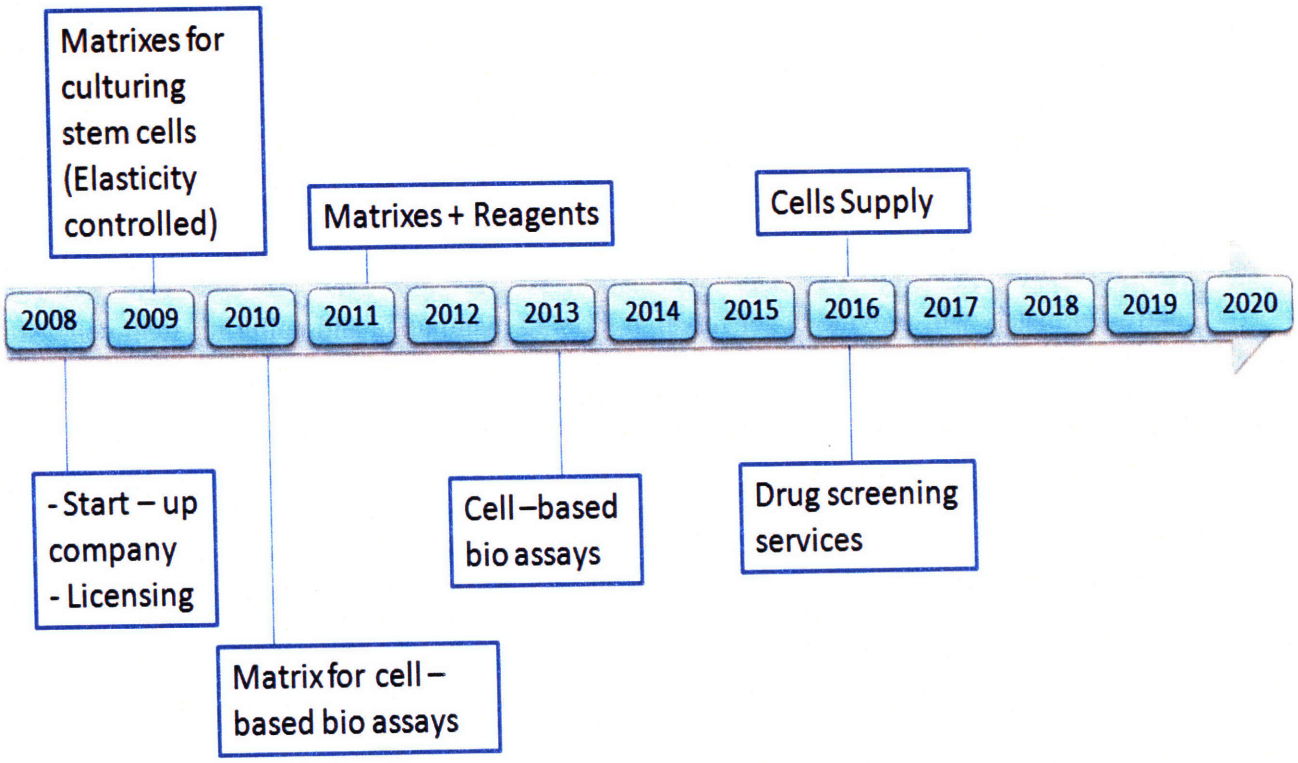


Figure 39: Business timeline

VIII. Conclusions

Stiffness-defined matrices have direct applications in stem cell research and drug screening market. Although these two markets are ill-defined, both of them are growing fast and appear to be very promising. A review of the technology itself led to the conclusion that the matrix is capable of induce anchorage dependent cell into specific lineage but the success rate is not yet quantified and further research need to be done to achieve good reproducibility and efficacy. During the process of creating a cost model for this technology and through learning from pre set-up companies such as Affymatrix, ESI, Promatrix, it became clear that the probability of commercial success for the matrix is from medium to high. It is feasible for us to set up a production company. On the other hand, there are still numerous challenges ahead. The two biggest challenges now are lack of clarity in IP landscape in the field and the need for long-time horizon funding.

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X. Appendix

MatTek Corporation

Customer Price List -Glass Bottom Culture Dishes

Product	*Dish Diameter/ Plate Type*	*Glass Thickness*	*Microwell Diameter (mm)*	*Coating*	*Sleeve Qty*	*Sleeve Price*	*Case Qty*	*Case Price*
	❶	❷	❸	❹				
Glass Bottom Dishes								
35 mm								
P35G-0-10-C	35 mm	No. 0	10	Uncoated	10	$40.00	200	$335.00
P35G-0-14-C	35 mm	No. 0	14	Uncoated	10	$40.00	200	$335.00
P35G-0-20-C	35 mm	No. 0	20	Uncoated	10	$48.00	200	$440.00
P35G-0-7-C	35 mm	No. 0	7	Uncoated	10	$45.00	200	$355.00
P35G-1.0-14-C	35 mm	No. 1	14	Uncoated	10	$40.00	200	$345.00
P35G-1.5-10-C	35 mm	No. 1.5	10	Uncoated	10	$40.00	200	$315.00
P35G-1.5-14-C	35 mm	No. 1.5	14	Uncoated	10	$40.00	200	$315.00
P35G-1.5-20-C	35 mm	No. 1.5	20	Uncoated	10	$48.00	200	$420.00
P35G-2-14-CGRD	35 mm	No. 2.0	14	Uncoated grid	5	$48.00	75	$450.00
P35GC-0-10-C	35 mm	No. 0	10	Poly-d-lysine	10	$48.00	200	$410.00
P35GC-0-14-C	35 mm	No. 0	14	Poly-d-lysine	10	$48.00	200	$410.00
P35GC-1.0-14-C	35 mm	No. 1	14	Poly-d-lysine	10	$48.00	200	$420.00
P35GC-1.5-10-C	35 mm	No. 1.5	10	Poly-d-lysine	10	$48.00	200	$390.00
P35GC-1.5-14-C	35 mm	No. 1.5	14	Poly-d-lysine	10	$48.00	200	$390.00
P35GCOL-0-10-C	35 mm	No. 0	10	Collagen	10	$48.00	200	$410.00
P35GCOL-0-14-C	35 mm	No. 0	14	Collagen	10	$48.00	200	$410.00
P35GCOL-1.0-14-C	35 mm	No. 1.0	14	Collagen	10	$48.00	200	$410.00
P35GCOL-1.5-10-C	35 mm	No. 1.5	10	Collagen	10	$48.00	200	$390.00
P35GCOL-1.5-14-C	35 mm	No. 1.5	14	Collagen	10	$48.00	200	$390.00
P35GTOP-0-20-C	35 mm	No. 0	20	Uncoated	10	$48.00	200	$500.00

Figure 40: Market's price (68).

Elasticity controlled Collagen coated Polyacrylamide Gel

Feedstock Prices

Collagen	945	$/g
Polyacrylamide	42	$/lot(1 lot: 1L)
Cross linking agent	15	$/g
Rejected raw material	-16	$/lot
lap equiments(tube, handles,...)	30	$/unit
Wrapping materials	0.3	$/m2

Operating Characteristics

Production Capacity	300000	lots/year
Anuual Production Volume	300000	lots/year
Lot Size (1m x 1m)	943.618778	dish/Lot
Operating Days	240	days / year
Operating hours	24	h/day
Exogenous Cost Factors		
Wage (including benefits)	25	$/hr
Electricity Cost	0.07	$/kWh
Equipment Life	10	yrs
Fixed Overhead	0.35	% of annual fixed cost
Building Costs	1000	$/m^2
Building Life	10	years
Equipment & Building Maintenance	0.25	% of equipment & building cost
Auxiliary Equipment Cost	0.2	% of equpment cost

Process Specifications

Polymerization

Cycle time	0.08333	hr/lots
Setup Time	0.75	hr/day
Unplanned Downtime	0.1	hr/day
Equipment Cost (fridge)	3000	$/equipment
Equipment Cost (mixer)	300	$/equipment
Equipment cost (Roller)	3000	$/unit
Water consumption	90	L/ lot
Energy	1.661833333	kWh/ lot
Space Requirement	40	m2
# Worker	0.5	#/hr

Collagen coating

Cycle time	0.08333	hr/ lot
Setup time	1	hr/day
Unplanned Downtime	1	hr/day
Collagen Solution consumption	0.0015	L/ lot
Collagen spreader	300	$/unit
equiment to make collagen coating unifo	150	$/unit

Equipment cost (Roller)	3000	$/unit
Energy	0.45	kWh/lot
Space requirement	40	m2
# Worker	0.5	#/hr

Cutting

Cycle Time	0.08333	hr/ lot
Setup time	1	hr/day
Unplanned Downtime	0.3	hr/day
Equipment cost (cutting machine)	1000	$ /cutter
Equipment cost (Roller)	3000	$/unit
Energy	0.5833	kWh/lot
Space requirement	40	m2
#Worker	0.5	#/hr

Quality Control

Cycle Time	1.66667	hr/ lot
Setup time	0.1	hr/day
Unplanned Downtime	0.05	hr/day
Equipment cost (Roller)	3000	$/unit
AFM Microscope+ integrated computer	200000	$/2 units
Energy	0.004999998	kWh/ lot
Space requirement	40	m2
# Workers	2	#/hr
Yield	0.8	

Packaging

Cycle Time	0.03333	hr/ lot
Setup Time	0.15	hr/day
Unplanned Downtime	0.07	hr/day
Equipment cost (Wrapper)	100000	$/unit
Equipment cost (Roller)	100000	$/unit
Wrapping materials	1.5	m2/lot
Energy	0.3066	kWh/ lot
Space requirement	50	m2
#Workers	0.75	#/hr